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ANNUAL REPORT OF PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

OCTOBER 1, 1977 THROUGH SEPTEMBER 30, 1978

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U.S. NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

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ANNUAL REPORT OF PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
OCTOBER 1, 1977 THROUGH SEPTEMBER 30, 1978

The Director completed and sent to the printer his second annual report entitled "A Matter of Opportunity." This report, designed for the lay reader and a very broad circulation, highlights selected scientific activities from intramural and extramural research programs. The organization of research activities into a program structure enables a more comprehensive reporting of scientific activities in the summaries of the Program Directors which follow. Accordingly, those sources should be sought for highlights of scientific progress.

A major activity of the Office of the Director during the year has been in relation to International Biomedical Research activities. The Director served on a White House Task Group assembled by the President's Assistant for Health Activities, Dr. Peter Bourne. The Task Group developed a comprehensive report which reflected all current U.S. governmental activities in the international health field and detailed needs. At the same time, the Office of International Health (OIH), DHEW, and the Institute of Medicine undertook reviews of U.S. involvement in international health and biomedical research activities, publishing reports and recommendations. Dr. Joseph Cook, as Special Assistant for International Biomedical Research, prepared much of the NIAID and NIH material utilized in those reports and participated in a number of the meetings where information was developed. Dr. Cook also was a principal NIH representative in two task groups appointed by the White House to develop a strategy document for the implementation of recommendations in the Bourne report. Internally, and in conjunction with the Fogarty International Center and the World Health Organization, an International Postdoctoral Fellowship Program has been developed and should become operational next year. This is targeted towards training scientists from developing countries for research on tropical diseases. Other initiatives are developing in the Microbiology and Infectious Diseases Program.

A second area of involvement has been with the DHEW Prevention Program. Dr. George Galasso served on a HEW Task Force to develop new or expanded initiatives in prevention. Following the development of a draft, the Deputy Director served on an NIH Committee that reviewed research initiatives. The final plan envisions expanded research in sexually transmitted diseases and in other infectious diseases where immunization should be effective, such as the acute, viral respiratory diseases.

The Deputy Director and several Institute scientists have been involved in a joint NIH-CDC effort to revise the publication "Classification of Etiologic Agents on the Basis of Hazard" and to bring this into compatability with the safe laboratory practices area of the NIH Guidelines for Recombinant DNA Research.

The Director has served or is serving on a number of committees, among them NIH Committees for the Development of Health Research Planning Principles, for review of the Fogarty International Center, and for NIH Policy on Safety. He also serves on the Interagency Diabetes Coordinating Committee and on an Office of Technical Assessment Committee for the Congress.

Speeches made by the Director - October, 1977 through September, 1978

1. Clinical Immunology. Prospects for Survival. October 5, 1977. Annual Meeting of the Society for Immunology, The University of Heidelberg, Heidelberg, Germany.

2. A Cartographer's Survey of Streptococcal Topography. November 7-11, 1977. Streptococcal Disease and the Immune Response Symposium, Trinidad.

3. The Interrelationships Between the Allergy and Immunology Societies and Government Agencies. November 10-11, 1977. Allergy Forum, New Orleans, Louisiana.

4. Science Knows No Country: The Contributions of the National Institutes of Health to Tropical Medicine Research. December 12-14, 1977. Anglo-American Conference on Tropical Medicine, London, England.

5. Research Challenges and Opportunities. March 6-8, 1978. Conference on Expanding the Role of Women in the Sciences, New York.

6. Advances in Allergy: The Residue or What Remains. April 1-6, 1978. American College of Allergists, 34th Annual Congress, Las Vegas, Nevada.

7. An Inquiry into the Cause of Pneumococcal Pneumonia: A Case History Concerning the Origins of Molecular Biology. April 12, 1978. Valparaiso, Indiana.

8. Recombinant DNA; A Technology with a Future. May 8, 1978. Washington Medical Surgical Society, Rive Gauche, Washington, D.C.

9. A Tale of Two Rivers; From the Ohio to the Potomac. May 11, 1978. Marietta, Ohio

10. Can Bilharzia Occur in the Delaware Water Gap? May 18, 1978. Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland.

11. Diabetes, Allergy and DNA or It's a Long Way from Amphioxus. May 31, 1978. The New England Society of Allergy, Weston, Mass.

12. Florence Nightingale: Midwife to Epidemiology. May 30, 1978. NIAID Symposium Impact of Infections on Medical Care in the U.S.: Problems and Priorities for Future Research, Bethesda, Maryland.

13. Lupus: The Revolt from Within. June 14, 1978. Banquet Address, Meeting of the Board, St. Louis Lupus Chapter, Clayton, Missouri.

Professional Societies and Agencies

Better interchange has been cultivated with those professional societies and voluntary agencies that relate to the Institute's work. This effort, begun when the present Director took office, has reached a more structured status. Two meetings were held with representatives of such societies as the Infectious Diseases Society, American Society of Microbiology, the four allergy societies, the American Thoracic Society, the American Dermatological Association, the Cystic Fibrosis Foundation and Lupus Foundation, and other groups. At these meetings, information on Institute research programs and initiatives was presented, the budgetary picture discussed, and views of the participants obtained on research needs.

Dr. Jack Whitescarver was appointed as a Special Assistant to the Director in July with a major responsibility of liaison to the professional societies and voluntary agencies. This provided a focal point in the Institute for relationships and for the interchange of information. Dr. Whitescarver's responsibilities extend to the Council and other advisory bodies.

The professional societies organized and held a banquet in late September to recognize the Institute's 30th anniversary as an Institute. This signaled one dividend of the improved interchange program. The societies have begun to work together to the common good.

Recombinant DNA Research

Dr. John Nutter, Head of the Office of Specialized Research and Facilities spearheaded an effort between NIAID, NIGMS, and NCI to develop and make available to the scientific community P-4 facilities. The first such laboratory in the United States was opened in Building 550 at the Frederick Cancer Research Center in March. A second facility in the renovated NCI mobile laboratory now situated at the NIH was opened in June. Additional interim facilities are under renovation in Bldg. 41, NIH, and are expected to open early in FY 1979.

Drs. Rowe and Martin of the Intramural Program began the first risk assessment study in Bldg. 550 about 10 days after it was opened. The long delayed experiment, given the highest priority by the NIH Recombinant DNA Research Advisory Committee, involves the insertion of polyoma virus genes into E. coli K12 through plasmid transfer and administration of the bacteria to mice. The question is that of expression of the virus gene in the animal host. Further details can be found in their individual project reports.

After their experiment was underway, an announcement was published on the availability of interim P-4 laboratories to investigators on DHEW grants and contracts beginning in early FY 1979. Priority will be assigned to applicants by the NIH DNA Recombinant Research Executive Committee.

A plan of requirements was developed for the National Biomedical Containment Laboratory. After a temporary delay while the needs for such a national P-3/P-4 level containment facility were reviewed by the Director, NIH in the light of the revision of Recombinant DNA Research Guidelines, an architectural and engineering contract was let for the renovation of Buildings 467, 468, 469 at FCRC. Construction contracts are expected to be effected in early calendar 1979 and the facility opened in 1980-81.

Meetings were held with the American Society of Microbiology Committee under contract to develop minimum requirements for biosafety training for principal investigators engaged in recombinant DNA research. This would be provided to Institutional Biosafety Committees as a guideline in judging the qualifications of independent investigators filing MUA's for research. We also expect to receive information on where or how the necessary training leading to qualification might be obtained.

Influenza

The Deputy Director has served with the Director, MIDP, on an interagency Task Group to develop HEW plans for the control of influenza. The Task Group completed a report which recommended that HEW undertake achievement of immunization of high risk persons on an annual basis. The Task Group report was available at the time that the occurrence of epidemics in Russia caused by an H_1N_1 strain of Influenza A virus was received. It was basically this report that enabled the HEW to quickly assemble and submit to Congress a request for supplemental funding to enable beginning such an annual influenza vaccine program in the summer of 1978.

The NIAID, Bureau of Biologics and CDC jointly supported several meetings to assess the data on H_1N_1 influenza and to discuss possible strategies applicable to the U.S. The three also planned the necessary clinical trials for candidate vaccine formulations. These were conducted by NIAID contractors involving about 3,000 individuals. Sufficient data had been accumulated and analyzed by July to enable the HEW Advisory Committee on Immunization Practices to make recommendations for vaccine dosage in those over age 13. Further data were accumulated on children 12 and under by late August, enabling recommendations for this age group. Studies in children under the age of 3 are continuing.

The NIAID also participated in the planning and conduct of an ad hoc Conference on Influenza held by the Secretary in August.

The Institute received an additional $1.2 million in a supplemental appropriation to pay the cost of clinical trials.

Technical Consensus Development Conference on Emergency Treatment of Insect Sting Allergy

On September 14-15, the Institute held a unique conference on the problems of emergency treatment of anaphylactic or severe reactions to insect stings. The primary issue was that of availability of commercially marketed insect sting kits containing injectable adrenalin to a broader community than that provided by physicians prescriptions to known sensitive individuals. There was a focus of advocacy for changes in federal regulations regarding the prescription status of adrenalin and for changes in state laws and requirements dealing with the practice of medicine, good Samaritan activities or other impediments to such availability. The conference was planned to review the problem of stinging insects, of known or projected statistics on severe or fatal reactions to stings, on present availability of emergency treatment to those most likely to be stung, and of past efforts to increase the availability of emergency treatment.

The format was appointment of a broadly based panel ranging from academic leaders in allergy to private practitioners from rural areas, public health nurses, lawyers, consumer groups, and volunteer first aid workers. The panel heard a series of presentations, including statements from apiarists, Boy Scouts, and other groups that might be benefited if emergency sting kits could be made available to them. They also heard legal and legislative opinions and of modifications of laws in the State of Connecticut toward increased availability.

At the termination of the meeting, the panel developed statements and recommendations on the problem and moves toward solution. Rejected out of hand was any concept of making kits an over-the-counter drug item. The thrust of recommendations was toward making them available to a limited number of specially trained individuals associated with the most "at risk" occupations or avocations. These reports will be widely published in appropriate medical journals.

Perhaps one of the most significant outcomes for the NIAID was the need for educational materials on insect sting for the primary care physician and for the public. Local allergy groups can be involved in the development and delivery of educational materials.

Equal Employment Opportunity

The year was a signal one involving the first full year of functioning of a revised EEO Advisory Committee. Operating under a new charter and by-laws, the Committee met with regularity, established subcommittees, and is beginning to provide substantive data and recommendations on EEO matters.

In December, two EEO conferences were held over a period of 4 days. Most of the staff attended, one-half for the first two days and the other half on the second two days. At each half of the conference, the Institute's Affirmative Action Plan was reviewed and discussed in detail. This conference was unique in NIH efforts and the NIAID received considerable credit for its efforts to make the Affirmative Action Plan a whole Institute activity.

Mr. Vincent Thomas, EEO Coordinator, also developed a plan for the EEO Team. The Team, consisting of the Director, Deputy Director, Executive Officer, Chairperson of the EEO Advisory Committee, and senior program officials is charged with implementing the program. By regular meetings with the EEO Coordinator, the Team reviews action items, accomplishments, problems, and new proposals or recommendations from the Committee.

Mr. Thomas was selected for university based management training and left his post in July. Recruitment of a replacement was successful in September when Mr. Franklin Fountain was appointed EEO Coordinator. He immediately undertook the review of the FY 1978 EEO plan with the Committee toward its approval by the Director before October 1.

Office of Research Reporting and Public Response

In an effort to heighten awareness among both scientific and lay groups of the impact of allergy and infectious diseases on the health of the American population, the Office of Research Reporting and Public Response (ORRPR) has increased and broadened its communications activities during the past year. These activities included active involvement in arranging, publicizing, and reporting Institute-sponsored scientific meetings, in addition to collaborating with voluntary and non-scientific groups to develop a more effective public education program.

Scientific Meetings and Information - Working closely with the scientific staff, ORRPR handled press relations and promotional materials for an NIH Consensus Development Conference on the Emergency Treat of Insect Sting Allergy held September 14-15, 1978. As the first of its kind sponsored by the NIAID, the meeting brought together technical experts, consumers, practitioners, and other occupations in the field of insect allergy to develop a consensus on whether changes in the current mode of management should be recommended.

In addition, the ORRPR assisted with publicity for two other NIAID scientific meetings held at the NIH: A Symposium on the Impact of Infections on Medical Care in the U.S.: Problems and Research Priorities (May 30-31, 1978) and a Workshop on the Origins of Inbred Mice (February 14-16, 1978)

Exhibits were developed by ORRPR for the following professional meetings: Seventeenth Interscience Conference on Antimicrobial Agents and Chemotherapy, New York City (October 12-14, 1977), Association of Military Surgeons of the U.S., Washington, D.C. (November 27-December 1, 1977), American College of Allergy, Phoenix, Arizona (February 26-March 1, 1978), American College of Allergists, Las Vegas, Nevada (April 1-6, 1978) and the American Thoracic Society, Boston, Massachusetts (May 14-17, 1978). Publications prepared by the ORRPR were displayed at the annual meeting of the American Society for Microbiology, Las Vegas, Nevada (May 14-17, 1978).

Seven articles have been prepared for the "News from NIH" section that appears monthly in the Journal of the American Medication Association. These articles, aimed at the general practitioners, report current research activities conducted or supported by the NIAID.

Twenty-two "Research Reports"--non-technical summaries based on papers or presentations made by NIAID scientists, grantees or contractors--were written during the year. These reports were distributed to the Institute's professional staff, advisory groups, science writers and to others on request. In addition to alerting these groups, the reports are valuable in replying to inquiries from Congress, the medical profession, press and general public.

Press Events--Major press briefings were held (1) to report the opening of the first certified P-4 maximum containment laboratory for recombinant DNA research in the U.S. (March 1978) and (2) to announce the results of the first controlled trial of the prevention of life-threatening allergic reactions to insect stings using venom extracts (July 1978). Both briefings were covered extensively by local and national media using film clips and background material prepared by the ORRPR. In addition, ORRPR prepared a news announcement on the use of an experimental pneumococcal vaccine for children with sickle cell disease.

Publications--ORRPR has created a scientific monograph series. The first volumes will contain the reports of two scientific panels, the Virology Task Force and the Asthma and Allergic Diseases Task Force. Both of these task forces were appointed by the Director, NIAID to review the state-of-the-art of these research areas. Lay versions are being written under contract and are expected to be published in early 1979.

The second issue of the Director's Report, "A Matter of Opportunity," written by the Director and Margaret McElwain, Assistant Chief, ORRPR, will be distributed to more than 15,000 medical and scientific professionals and representatives of selected organizations. In addition, Mrs. McElwain has prepared an Institute brochure describing current research programs of the NIAID.

During the past year ORRPR prepared new fact sheets on "Staphylococcal Infections," "Dengue," and "Acne," and revised the following: "Viral Hepatitis," "Asthma," "Sexually-Transmitted Diseases," "Malaria," and "Questions and Answers on Recombinant DNA Research." Reprintings were ordered for "Q and A on Allergies," "Pneumococcal Infections," "Hospital-Associated Infections," "Tuberculosis," "Mononucleosis," and "Sarcoidosis." Approximately 225,000 NIAID publications were distributed during the year.

ORRPR also assisted in the publication of the following NIAID publications for distribution to medical and scientific professionals: NIAID Catalogue of Tissue Typing Antisera Supplement and Proceedings of the Thirteenth Joint Cholera Conference.

Public Information and Education-ORRPR produced a 10-minute slide-tape presentation on "What You Should Know About Asthma" directed at patients with asthma and their parents. The enthusiastic response to the presentation encouraged mass production of the slide show, which will be available for purchase from the GSA at a nominal fee beginning October 1, 1978. Working with the local chapter of the Allergy Foundation of America, ORRPR is currently planning a slide tape presentation to alert school officials and parents on the problems of allergic children in the school environment.

In similar efforts aimed at increasing public awareness, ORRPR staff contributed to public information activities at the annual meeting of the Juvenile Diabetes Association, Washington, D.C. (June 1978) and provided publications at local meetings of the Allergy Foundation of America. Two members of the ORRPR staff also served on an HEW Task Force to discuss new approaches to improving public education on sexually-transmitted diseases.

Public inquiries constitute a major portion of the work of ORRPR. In the past year, more than 600 letters and 8,100 phone calls from the public required special handling. Responses to public inquiries are coordinated by Mrs. Jeanne Winnick, Information Assistant, who received the NIH Merit Award for "high level performance, initiative and persistence in responding to public inquiries received by the NIAID."

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Immunology, Allergic and Immunologic Diseases Program
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REPORT OF THE DIRECTOR

IMMUNOLOGY, ALLERGIC AND IMMUNOLOGIC DISEASES PROGRAM

Fiscal Year 1978

A. Administrative Summary

1. Organization and Function

The Immunology, Allergic and Immunologic Diseases Program (IAIDP) was initiated on October 1, 1976, coincident with the reorganization of NIAID into major programs characterized by professional function. With this change the IAIDP assumed the responsibility for research, training, and conference activities that formerly were assigned to the Allergy and Immunology Branch of the Extramural Programs and contract activities in transplantation immunology previously administered within the Collaborative Research Program.

During FY 1978, the following organization of the IAID Program was developed:

Office of the Director

Director-Sheldon G. Cohen, M.D.
Special Assistant to the Director-Dorothy D. Sogn, M.D.
Assistant for Program Management-George R. Jenkins, B.S.
Collaborative Projects Section-Daniel I. Mullally, M.D.

Allergy and Clinical Immunology Branch

Chief-Robert A. Goldstein, M.D., Ph.D.

Immunology and Immunochemistry Branch

Chief-Bernard W. Janicki, Ph.D.
Program Officer-Henry Krakauer, M.D., Ph.D.
(Washington State University, IPA position)

Genetics and Transplantation Biology Branch

Chief-Carl Cohen, Ph.D. (University of Illinois, IPA position)
Program Officer-John G. Ray, Jr. Ph.D.

Each of the three IAIDP Branches assumes responsibility for administering investigator initiated research grants and collaborative research contract awards, developing programmatic initiatives and new projects, serving on trans-NIH coordinating committees and taking active roles in the development and conduct of workshops and conferences in areas of the biological disciplines and medical specialties with which they are concerned.

All three Branches are also involved with programs related to Research Career Development Awards and training, i.e., individual and institutional fellowships. In addition, the Allergy and Clinical Immunology Branch administers Allergic Disease Academic Awards which are designed to further the careers of mid-level investigators preparing for careers in academic allergy research and the Young Investigator Research Grant Awards which are designed to assist and encourage investigators in early stages of their careers to develop research interests and capabilities in clinical aspects of immunology.

The IAID Program also assists investigators in their research by providing certain reagents that are not otherwise available from commercial sources. Such reagents are intended either for reference standard purposes or to supply working quantities of unusually rare or expensive materials for studies related to program activities. Included are materials such as human and mouse histocompatibility typing sera and various purified allergens. Program staff has the responsibility for maintaining adequate inventories of reagents as well as for developing new sources and identifying new materials for acquisition.

A new Program activity developed in FY 1978 was the establishment of a pilot program of Centers for Interdisciplinary Research on Immunologic Diseases (CIRID). Four Centers have been established at an approximate total first-year cost of $1,225,000. This program was developed by staff following Congressional encouragement to develop a regionally-based effort to accelerate clinical application of new knowledge of the immune system. The new Centers are located at the University of California in Los Angeles, Washington University in St. Louis, Georgetown University in Washington, D.C., and at the University of Rochester. These activities are designed to favor integration and coordination of research projects in clinical immunology being pursued in other clinical specialties (e.g., dermatology, pulmonary medicine, hematology, nephrology, rheumatology, infectious diseases and otorhinolaryngology) with those in basic research (e.g., immunobiology, immunochemistry, microbiology, virology, genetics, biochemistry, pharmacology, general physiology and pathology). Study of one or more allergic diseases is a necessary component of a Center's program in immunologic diseases. Within the research framework Centers will develop special projects and activities, e.g., recruitment and training of clinical investigators, demonstration programs, assessment of regional socioeconomic impact of immunologic and allergic diseases, evaluation of new treatment modalities, application of promising investigative findings, and continuing medical education of practicing physicians. An indepth review of progress is scheduled after the Centers have functioned for three years to determine future directions and if expansion of the program is justified. IAID Program staff has the responsibility for monitoring the activities and progress of the Centers during this period.

2. Budget

The following shows the distribution of support by award mechanism for the activities of the Program during FY 1978.

Immunology, Allergic and Immunologic Diseases Program
FY 1978 Awards

Award Mechanism		Number	Amount[1]
Research Grants		359	$37,026,294
Career and Career Development Awards		57	1,965,070
	Subtotal	416	$38,991,364
Fellowship Awards		67	828,050
Training Programs		33	2,229,494
	Subtotal	100	$ 3,057,544
Contracts		51	3,035,399
	Total	567	$45,084,307

[1] Total costs, estimated using a 40% overhead for indirect costs.

3. Conference Support

During FY 1978, the following meetings, conferences, and workshops relevant to the activities and functions of the IAID Program were supported.

October 77
- Third HLA Workshop of the Americas, Kansas City, MO.
- Post Forum of the Seventh International Histocompatibility Workshop, Kansas City, MO.

December 77
- Asthma and Allergic Diseases Centers Workshop, Bethesda, MD.
- Escape from Immune Surveillance: The Interface Between Immune Mechanisms and Diseases Conference, Brook Lodge, MI.

February 78
- Conference on Transmembrane Signaling, Keystone, CO.

March 78
- Conference on Hematopoietic Cell Differentiation, Keystone, CO.
- A Workshop on In Vitro Induction and Measurement of Antibody Synthesis in Man, Utrecht, Netherlands.

May 78
- Immunogenetics of the Rabbit Conference, Bethesda, MD.
- H-2 Mutation Conference--A Tool in Immunology, Woods Hole, MA.
- Fourth Ir Gene Workshop--Aspects of Specific Immune Genes and Their Products, Annapolis, MD.

June 78	Third American Histocompatibility Workshop, Boston, MA. Twelfth International Leukocyte Conference, Beersheba, Israel. Cytomegalovirus Conference, Bethesda, MD.
July 78	Gordon Conference on Immunobiology and Immunochemistry, Proctor Academy, NH.
September 78	International Congress of the Transplantation Society, Rome, Italy. Third Conference on Mononuclear Phagocytes, Leiden, The Netherlands.

B. Scientific Summary

The Immunology, Allergic and Immunologic Diseases Program (IAIDP) is primarily concerned with the immune system as it functions in the maintenance of health, as it is involved in immunologic, hypersensitivity and allergic diseases, and with its genetically conditioned role in transplantation biology and transplantation surgery. Immune structure and function are of programmatic interest in our concern with the basic biology of the system and applied studies are included within our programmatic activities in allergy, diseases of the immune response system and tissue transplantation. Our responsibility for work in these biomedical areas is effected through program activities concerned with investigator initiated research projects, collaborative contracted research and development studies, NIAID Asthma and Allergic Diseases Centers (AADC), NIAID Centers for Interdisciplinary Research on Immunological Diseases (CIRID), Lymphocyte Biology program projects, development of research resources, and procurement and distribution of standard reference research reagents and materials including the maintenance of a serum bank for histocompatibility testing.

To meet these objectives the IAIDP has given its attention and concern to three relevant major biomedical and scientific areas reflected by the work of the Program Branches to whom the respective responsibilities have been given: Immunobiology and Immunochemistry, Allergy and Clinical Immunology and Genetics and Transplantation Biology. The summary that follows is intended to present an overview of emerging developments and potentially important aspects of ongoing research in the IAIDP's sphere of direct interest and concern. Basic studies in immunobiology, immunochemistry and immunogenetics are reviewed both for the fundamental knowledge generated and for the potential applications of basic research data to problems concerned with health and disease. By keeping attuned to clinical research in asthma, allergy, clinical immunology, immunopathology and transplantation, we seek to enhance the prompt translation of investigational needs to improved methods of diagnosis, prevention and treatment of allergic and immunologic disorders.

Studies of the ontogeny of immune cells are beginning to define the pathways of flow from stem cells to effector cells. Research has reached the point where T and B lymphocytes can be identified as stem, progenitor, precursor and functioning cell forms by means of serologically identified cell

surface properties as well as by means of physical properties. As a result, it is now possible to localize certain defects in immune function and identify points of block corresponding to specific immunodeficiency patterns. Application of these findings is anticipated to lead to the manipulation of feedback mechanisms and to the treatment of failures of immune function, for example, with thymic hormones. Similar techniques are now evolving for tracing pathways of immune development in the fetus through liver to bone marrow. This may offer the opportunity to reconstitute immune deficiencies with fetal tissue to avoid graft vs. host reactions. Of importance will be the ability to establish relationships between age of the fetus and specific ability to recognize antigens so that reconstitution by fetal cells of appropriate age and development may result in desired tolerance of the recipient patient. In the case of pathways of differentiation of antibody producing cells destined to secrete the various immunoglobulin isotypes, potential significance in applicability remains to be evaluated. Studies of cell-to-cell interactions among B lymphocytes indicate the existence of feedback mechanisms that result in the suppression by IgG secreting cells of the earlier appearing IgM secretors. Such studies increase our understanding of the critical phenomenon of autoregulation.

Studies of thymus derived lymphocytes have revealed the dominant role of these cells in control processes. They possess helper, suppressor and effector functions and thus modulate the responses of both antibody producing cells and of the other effector T cells. Thus we are now in a position to deal selectively with the study of specific cell functions. This emerging knowledge may be applied to understanding and hopefully attacking those human diseases in which the barriers to reaction to self, which are perhaps based on recognition and specific suppression of reaction to self, are breached. Here productive and revealing studies on another cell involved in antigen processing, the macrophage, have become important in unraveling mechanisms of cell-to-cell signals of communication.

Determining the expression of properties of the immune system encoded in genes, being approached and aided by the tools of molecular biology including highly efficient DNA sequencing methods and recombinant DNA technology, will permit the enumeration of the genes and will result in the unraveling of the mysteries of their organization and the controls of their expression. Indicators point to the pre-existing availability of all genes and to the likelihood that antibody diversity does not have to arise by mutation.

There is, however, an evident gap between the forefronts of research in immunology and molecular biology in that information is needed on the biologic processes occurring within functioning immune cells. Attempts should be made to encourage work focused on intracellular processes that generate signals and result from their reception.

Techniques utilizing hybridomas, myeloma tumor and immunoglobulin producing cell hybrids are being developed for studies of or dependent on antibodies. The technique of cell fusion and selection allows for the insertion of antibody producing characteristics from an antigen stimulated cell into appropriately cultured myeloma cells. At this time this in vitro methodology serves as an effective tool for obtaining monoclonal immunoglobulins for

studies with antibodies especially those specific for particular cell surface antigens, e.g., receptors for hormones, thus facilitating the study of endocrine physiology. Another related technique, somatic cell hybridization with chromosome deletion, is being used to localize the genes for the major histocompatibility antigens.

Current immunochemical studies are directed principally to the chemical characterization by sequencing and x-ray crystallography of proteins characteristic of the immune system. One area of prominence in the current chemical structure studies is that dealing with cell surface antigens. There is an evident need for increased understanding of the molecular organization of the cell surface and of its reorganization in the course of functional alterations induced in the immune lymphocytes. A rapidly developing area in the study of immune function is one involving the genetic relationships pertaining to immune responsiveness. Genetic control of immunoglobulin synthesis is not the sole province of the immunoglobulin genes. Regulatory functions have been also mapped in the major histocompatibility complex located on a separate chromosome and have been designated Ir genes. The detailed investigation of the functions of these genes has led to the potentially seminal concept that recognition of self and the absence of reaction to self may occur by suppressive activity of differentially stimulated cells.

Expanding knowledge of immunogenetics has arisen out of results of tissue typing for transplantation. A predominant role in determining histocompatibility is played by specific chromosomal regions called the major histocompatibility complex (MHC), designated H-2 in the mouse--the most thoroughly characterized animal model--and HLA in man. Certain cell-surface antigens are produced by genes within the complex. They are commonly identified by testing against cytotoxic antibodies specific for them. Other antigens result in the activation of nonsyngeneic lymphocytes (LAD's). These are identified in mixed lymphocyte culture (MLC) and also map in the MHC. Associated with them are serologically identified antigens found primarily on B cells. These progressive refinements of our understanding of the histocompatibility genes mark a very significant advance of recent time. Thus, matching donors of grafts and their recipients for these antigens is very useful if donor and recipient are related. If the donor is the parent or a sibling of the recipient, serological matching at the HLA A and B loci results in a 3-year kidney graft survival probability of over 80 percent compared to a 35 percent probability if there is a complete discordance. On the other hand, when donor and recipient are not related such dramatic improvement due to matching is not achieved. This represents, therefore, an area where continued intense effort is required. The barriers to success are both theoretical and practical. The number of histocompatible loci is large, as is the polymorphism of the alleles at each locus. Consequently the probability of at all adequately matching unrelated donors and recipients is low and that of finding a matched donor at the time of need by the recipient is even lower. Improvements in transplantation require, therefore, reliance also on strategies other than histocompatibility typing.

A mainstay of current methodology is immunosuppression with cortical steroids and cytotoxic agents. These drugs carry a heavy burden of side effects, including the inevitable one of destroying resistance to infection.

Better methods are constantly sought. A more refined and selective approach, the use of antilymphocyte, or better yet antithymocyte antisera, to destroy the cells responsible for graft rejection, has been successful in animal models. However, its promise has not yet been fulfilled in man. Another promising approach tested successfully in an animal model, the dog, and considered for human use, is "lethal" total body irradiation in preparation for grafting to destroy all lymphoid tissue, and reconstitution with autologous bone marrow. All of these effects are marked by ever increasing insight and discretion in the application of the methodologies being provided by basic research. It is now possible to monitor the development of rejection activity by means, for example, of the appearance of cells that synthesize DNA at a high rate and to abort such activity with carefully tailored immunosuppressive therapy with either cytotoxic agents or anti-lymphocyte serum. Measures of the effects on graft survival of other parameters such as age, latent infection with cytomegalovirus, manner of handling and storage of the graft, immunologic status and manipulation of the recipient are being developed. It is, thus, hoped to attain by means of cumulative improvements, in many facets of transplantation, major overall benefits to the patient receiving a graft.

In recognizing the needs of patients afflicted by asthma, allergic disorders and immunologic diseases, there is an appreciation of the potential for attacking pertinent questions that can be found and translated from ongoing basic research and clinical investigation. To identify these areas, the Institute is sponsoring a Task Force on Asthma and the Other Allergic Diseases. Its report is designed to describe the state of knowledge, incidence and impact of these diseases and to define research objectives which may be expected to advance basic understanding, diagnosis, prevention, and therapy of these diseases. Accordingly, conditions are now even more favorable than before to seize the opportunity for influencing and supporting research in some specific areas summarized below.

Asthma: Continuing contributions resulting from collaborative efforts of research allergists working with pulmonary physiologists, pharmacologists, immunologists, and other basic scientists dealing with infection, biochemistry and genetics verify the impression that asthma is a disease resulting from several interacting influences. Clues to the multifactorial origins of asthma are likely to come from studies in viral infection, chemical mediator release, neurogenic elements and a variety of inflammatory agents that may play associated roles in disease development in people genetically predisposed to allergy. Analysis of incoming information indicates that extension of ongoing research in subject areas that follow can be looked upon as promising and potentially important.

The role of IgE mediated mechanisms in long term nonspecific hyperreactivity of the bronchi requires delineation in the same terms as the function of allergic mechanisms in immediate bronchial reactions. The ability to prevent mucus production and edema of bronchial walls will depend upon studies of the effect of inflammatory factors in normal bronchial physiology. Increasingly, chemicals are being identified in causing occupationally incurred asthma. Thus, studies on the physicochemical definition of antigen

in the induction of sensitization are being pursued. Neuropharmacologic investigations present evidence of the existence of neurally mediated bronchial reflexes. Hence, both the adrenergic and recently described nonadrenergic system need further definition especially to understand and effect bronchial relaxing mechanisms. Based upon the productive biochemical studies identifying and characterizing both mast cell products and complement derived inflammatory agents, it may now be possible to develop specifically designed pharmaceutical agents to act at identifiable points along both immune and physiologic pathways.

Drug Reactions: The high incidence of untoward reactions to drugs in patients under treatment for a variety of diseases continues to be a major problem. Since development of new therapeutic approaches will be based upon an understanding of the mechanisms of allergic drug reactions, the following experimental approaches are considered important: studies on drug metabolism to differentiate sensitization to drugs as a function of metabolic handling in contrast to those that result from true immunologic mechanisms; elucidation of the chemical structure of that portion of the drug molecule responsible for allergic reactivity; and predictive tests and synthesis of nonsensitizing drug analogues. Because of the lack of suitable methods for predicting drug allergic reactions, especially with low molecular weight substances, further efforts will be directed to developing reliable in vitro methods. Similarly, in order to minimize risks of administering potentially allergenic drugs to humans, it will be necessary to work to develop suitable animal models for laboratory testing.

Allergic dermatitis: Studies directed to identifying disorders and their mechanisms where skin may be primarily involved as target tissue by immuno- humoral and/or cellular reactants, or where cells of the integument may serve as natural sources of specific antigens in immune processes are giving promising leads for the delineation of those based on IgE mechanisms, complement activation and deposition of immune complexes: e.g., erythema nodosum and multiforme, the blistering diseases (pemphigus, bullous pemphigoid, dermatitis herpetiformis, herpes gestationis) lupus erythematosus, viral exanthems and vasculitides. In atopic dermatitis, definition of possible interacting etiologies in this complex disorder points to the newly identified problems in T cell abnormalities. Further work is proceeding on genetic predisposition, IgE responses and cyclic AMP cell biologic functions--all of which may influence the development and course of this multi-factorial disease.

Urticaria and angioedema: As a result of relevant biochemical and immunologic studies, it is becoming increasingly clear that a common denominator may not exist in the disorders which present clinically as cutaneous edema. Extended work is continuing on the role of chemical mediators of vascular permeability released during inflammatory responses involving immediate hypersensitivity and complement. Accordingly, it may be possible to design corresponding approaches to treatment with specifically formulated pharmacologic agents. The demonstration that actual lesions of angiitis appear not only in the disorder of palpable purpura but also in recurrent urticaria has led to the identification of the role of streptococcal and hepatitis B antigens in clinically relevant syndromes.

Mechanisms of Allergic Disease: The attack on problems of certain immune complex and autoimmune mediated diseases by pertinent studies concerned with basic biology of the immune system is being extended for potential applications to immediate hypersensitivity disorders. The isolation and description of IgE has offered substantive leads for research demonstrating the manner by which its localization on mast cell receptors triggers release of inflammatory mediators of vascular permeability, bronchospasm and eosinophil migration. As a result, specific pharmacologic agents may now be designed to both inhibit the release of these mediators and modulate their tissue effects. In addition, with identification of platelets as the source of some potent chemical mediators it may be possible to utilize pharmacologic approaches to prevent platelet aggregation and mediator release.

To achieve these objectives we are supporting studies on IgE chemistry and function designed to define structural molecular characteristics of basophil and mast cell receptors for IgE and to develop the methodology necessary to block IgE fixation. Regarding the regulation of IgE synthesis and secretion, there are studies underway designed to develop information on the genetic control of the quantity and quality of reaginic antibody response and its cellular regulation by thymus dependent (T) lymphocytes. In this connection, it is pertinent to note preliminary data suggesting that immunoglobulin (IgE) responses associated with certain tissue invasive parasitic helminth infections may influence allergic IgE mediated disorders by occupying cell receptor sites. Studies of eosinophil biology and eosinophil-mast cell relationships are providing insights into possible definitive roles for the eosinophilic leukocyte in hypersensitivity disorders. Chemical definition of eosinophil products further indicates that this cell may be involved in reparative processes effected by mast cell derived chemical mediators. In the study of immediate hypersensitivity reactions associated with helminth tissue infections, the schistosomula model is of unique interest, pointing out the potential of eosinophil migration and chemical products for cytotoxic attack on invasive organisms.

Diagnosis and therapy of allergic disorders: To expedite advances in improved methods for diagnosis and treatment of allergic diseases, work is being concentrated on the development of appropriate reagents and methods. The isolation, identification and preparation of purified and standardized ragweed and grass pollen extracts have made it possible to further definitive research on problems related to etiologic mechanisms and treatment of seasonal rhinitis and allergic asthma. Similar work on hymenoptera derived venoms have made it possible to pinpoint anaphylactic reactors and provide effective immunotherapeutic treatment procedures for the prevention of potentially fatal sting reactions. Based on this experience, it is evident that similar approaches should be extended to other still uncharacterized allergens. Investigators with the requisite chemical expertise are being encouraged to undertake studies on molds in order to expedite definitive work on the immunopathology of hypersensitivity pneumonitis. There is also evident need to work out the etiology and pathology of a large group of gastrointestinal disorders which may be immune related, e.g., eosinophilic gastroenteritis, Crohn's disease and ulcerative colitis. Until complex foods have been fractionated into isolated and identifiable antigenic components for

study, there can be little progress in our understanding of food allergy. In addition, studies to differentiate allergenic and immune related mechanisms from those due to gastrointestinal enzymatic deficiencies and disorders in metabolic processing will be enhanced by the availability of purified food allergens. Design and development of in vitro techniques for identification and quantitation of antibodies of the IgE class will be required. Such in vitro testing must be correlated with allergic manifestations. It is encouraging that preliminary studies employing chemically modified allergenic extracts indicate that immunotherapy with improved safety and therapeutic results over shorter periods of time may be attainable. However, further work along this line especially designed to induce tolerance by manipulation of thymus dependent (T) lymphocyte controlling mechanisms appears to be the most hopeful approach to the specific treatment of allergic diseases.

Immunodeficiency disorders: While basic immunologic research has done much to define defects in pathways of cellular differentiation to explain congenital immune system dysfunction, additional work will be required to understand the basis of acquired immunodeficiency states. Studies are needed to explain the anergy associated with tumors and certain infections, and the hypogammaglobulinemias acquired after certain viral infections. Host factors altering expression and favoring susceptibility of immunodeficient and immune suppressed patients to cytomegalovirus, fungal and Giardia infections are needed to assist efforts in the diagnosis and treatment of these diseases. In the area of reconstitution of immune deficient patients, attempts to perfect techniques are under way. Of particular interest are the approaches utilizing enzyme replacement or stimulation and the preliminary success achieved with cultured thymus epithelial cells aimed at eliminating passenger lymphocytes responsible for the graft vs. host reaction.

Immune complex and autoimmune disorders: Research on the many relationships between T cell immune phenomena and diseases caused by intracellular parasites are providing preliminary data to suggest that T cell recognition and association with major histocompatibility complexes could possibly link viruses to causation of certain autoimmune diseases. This could well involve cell-specific surface determinants that serve as receptors for differentiation signals, e.g., triggering antibody production in B cells, suppressing activation of T cells, shifting production of IgM to IgG in B lymphocytes, and enhancing production of proteolytic enzymes in the macrophage. The role of anti-receptor antibodies is also of great interest especially since three diseases have been identified with antibodies to receptors on cell membranes; hyperthyroidism, myasthenia gravis and cases of insulin-resistant and juvenile diabetes. Others likely exist and antibodies to receptors for hormones have considerable potential especially in inhibiting the action of hormones by blocking the receptor. The detection of anti-islet cell and anti-insulin antibodies are also contributing to our understanding and management needs in patients affected by the diabetic spectrum.

Inflammation: Studies on the basic biology of complement and on the triggering and release of leukocyte derived chemical mediators have shed considerable light on the immunopathology of inflammation. Relevant work directed to their clinical application has a place in our need to improve

diagnosis, prevention and treatment of the patients affected by hypersensitivity disorders. In studies on further delineation of the complement system, especially important is that of nonimmunologic stimulation of the alternate pathway for its correlation with disease processes. The observation that mast cell mediators can be released by nonimmunologic means and that mediators of inflammation can be released by polymorphonuclear neutrophilic leukocytes as well as mast cells and basophils can be usefully applied in our differentiation of those inflammatory disorders that simulate allergic processes. The work on transfer factor (TF) that initially showed great promise in its application to disorders characterized by anergy loses its impact in newer data that suggest that possible TF effects may arise by nonspecific adjuvant means. Specific studies on chemical characterization and *in vitro* assay of TF should be encouraged since this information is prequisite to further clinical investigations on such conditions as oromucocutaneous candidiasis and osteogenic sarcoma.

ALLERGY AND CLINICAL IMMUNOLOGY BRANCH

A. Scope

This Branch is concerned with the etiology, pathogenesis, diagnosis, prevention and treatment of both naturally occurring and acquired allergic and immunologic diseases.

Studies on asthma include factors, both primary and predisposing, which contribute to the production of asthma, such as extrinsic allergy, infection, abnormalities of the sympathetic nervous system, cellular and chemical mediators of inflammation, and pharmacologic agents.

Relevant studies of allergic diseases supported by the Branch include: (1) immediate type hypersensitivity and its disorders (allergic rhinitis, atopic dermatitis, urticaria and angioedema); (2) allergic phenomena affecting respiratory gastrointestinal and cutaneous tissues; (3) allergic reactions and disorders caused by insect bites and stings, foods, airborne allergens, and infectious agents; (4) humoral antibody, particularly IgE, and the chemical mediators released by the interaction of antigen and antibody on target cells; (5) therapy and prevention of allergic disorders and hypersensitivity reactions by immunotherapy with specific antigens or drugs; (6) manifestations of delayed hypersensitivity and contact dermatitis; (7) mechanisms of drug reactions and chemical sensitization; and (8) isolation and chemical characterization of the active fractions of allergenic agents.

In the area of immunologic diseases, the Branch activities are focused on studies of the underlying mechanisms of disease and the application of basic knowledge to the etiology, prevention and management of immunologic disorders. Either or both of two disciplinary approaches, Clinical Immunology and Immunopathology, are involved in this effort. Studies in Clinical Immunology are directed toward acquired and inherited diseases associated with dysfunctions of the immune system. Immunopathology studies include genetics, cytology, biochemistry, physiology and pharmacology of the immune system. Relevant areas supported by the Branch include studies of (1) immune deficiency diseases arising from primary defects in development or maturation of the immune response or secondarily resulting from disorders affecting immune responses, (2) clinical manifestations mediated by products of lymphocytes, (3) diseases associated with immune complexes and autoimmune phenomena, (4) immunodermatology, i.e., immune disorders involving the cutaneous system, and (5) immunotherapy of disease processes.

The ultimate goal of the Allergy and Clinical Immunology Program is to promote the acquisition, translation, and application of research findings to the diagnosis, prevention, and treatment of allergic and immunologic diseases. To achieve this goal, the Branch is responsible for and manages Programs of Institute Emphasis (PIE) in Asthma and Allergic Diseases, Immunologic Diseases, and Immunology Centers and Program Projects. To support this effort, the Branch also obtains and distributes selected research reagents and materials to investigative allergists and clinical immunologists.

B. Awards and Support Levels

Relevant activities are supported by the Branch through various mechanisms including contracts, individual post-doctoral fellowships, institutional pre- and post-doctoral training grants, career and career development awards, as well as investigator-initiated research grants.

The following shows the distribution of support by award mechanism for the activities of the Branch during FY 1978.

Allergy and Clinical Immunology
FY '78 Awards

Award Mechanism	Number	Amount[1]
Research Grants	131	$14,445,518
Career Awards and Career Development Awards	24	872,724
Subtotal	155	15,318,242
Fellowship Awards	19	246,468
Training Awards	25	1,619,088
Subtotal	44	1,865,556
Contracts	4	335,562
Total	203	17,519,360

[1] Total costs, estimated using a 40% overhead for indirect costs.

The distribution of these awards by area of study is approximately 40% for asthma and allergic diseases and 60% for immunologic diseases. Approximately 1/3 of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

Support for the PIE activites is included in the research grant category. During FY 1978, the Branch supported these activities with awards for 13 Asthma and Allergic Disease Centers at a total cost of $1,720,522, 9 Clinical Immunology and Immunopathology Program Projects at a total cost of $3,611,861, and 4 Centers for Interdisciplinary Research in Immunologic Diseases at a total cost of $1,225,586.

C. Program Areas and Highlights

1. Asthma and Allergic Disease Centers (AADC) Program

A network of 13 asthma and Allergic Disease Centers, diverse in both its geographic and scientific breadth, is actively engaged in a collaborative fashion to gain new knowledge and insights into the field of hypersensitivity. Research, both fundamental and applied, is conducted; and, through this multifaceted approach, methods have already been developed and applied to improve the diagnosis and treatment of asthma and other allergic

diseases. While the emphasis of the Centers program is directed toward a better understanding of the mechanisms involved in the various allergic diseases, the effect in terms of better patient care is evident.

The activities of individual Centers participating in the Program are diverse, but so are the diseases themselves. While a few Centers concentrate their efforts on only one disease, most are studying several different allergic disorders. The scope of investigations encompasses a broad probing of basic cellular and humoral mechanisms underlying hypersensitivity for the development of better diagnostic and immunoprophylactic measures directly applicable to a particular allergic disease. Although all of the Centers serve as referral centers for patients with allergic diseases in their geographic areas and all are active in training future academic and investigative allergists, each of the Centers has also been successful in competing for additional support through either the regular grant program, training grants, or research career awards.

The AADC Workshop, Bethesda, December 1-2, 1977, was the seventh annual meeting of the directors and professional staffs from the 13 Asthma and Allergic Disease Centers supported by NIAID. Over 100 participants met at NIH to review recent progress by the grantees in this Center's program and to exchange scientific information in this dynamic field. More than 20 papers were presented. Among the topics discussed were the role of the mast cell in allergic diseases, mediators of allergic responses, urticaria and angioedema, mechanisms of reactions to radiographic contrast media, and the immune response to hymenoptera venom antigens.

2. Mechanisms of Allergic Diseases

Studies within this program have been concerned with investigations of the etiologic factors, pathogenetic mechanisms, diagnostic measures, and therapeutic approaches to the effective management of various allergic disorders.

The release of inflammatory molecules from tissue mast cells is a necessary step in the evolution of allergic reactions of the immediate type. Interestingly, recent work indicates that release of these mast cell mediators may be important in other immune reactions such as graft rejection, poison ivy or immune diseases of the kidneys. Timothy J. Sullivan (AI 00190, Washington University) has studied the mechanisms by which mast cells regulate their own secretion. He found that intracellular levels of cyclic-3', 5'-adenosine monophosphate and extracellular levels of adenosine have potent influences on mast cell secretions. These findings have opened the way to the deliberate development of drugs capable of efficiently and hopefully selectively controlling mast cell mediated inflammation. Kimishige Ishizaka (AI 10060, Johns Hopkins University) has also looked at the mechanism of mast cell secretion. His findings indicate that polymerization of cell-bound IgE is not an essential process of triggering and that local changes at the cell membrane, induced by bridging of receptor molecules, or interaction between adjacent receptor molecules is responsible for triggering mast cell secretion. Another of his experiments indicated that antigen-antibody reactions on mast

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cells do not necessarily induce histamine release unless certain membrane components are involved in the reactions, and that the IgE receptor is one of the limited components. Lawrence Lichtenstein (AI 11223, Johns Hopkins University) has been concerned with the pharmacologic control of inflammatory cells including basophils through hormone-receptor interactions. He has established that these cells have specific receptors for histamine, catecholamines, prostaglandins, adenosine, and other biologically active molecules. The release process generates mediators such as histamines or prostaglandins during inflammatory reactions which then control or modulate the effector function of the inflammatory cells.

Stephen Wasserman (AI 00254, Harvard University) has isolated arylsulfatases A and B, two enzymes which destroy slow reacting substance of anaphylaxis from rat mast cells and leukemic basophils. These enzymes are released from the mast cells by two separate pathways. Andrew J. Grant (AI 12621, University of Texas Medical Branch, Galveston) has looked at histamine release from basophils and has found that C5a, a small fragment from the fifth component of the complement system, binds directly to the blood basophil and induces its histamine release.

The role of reaginic antibody (also known as IgE) which circulates in the blood is well established in the allergic response. Frederick J. Grundbacher (AI 12360, Peoria School of Medicine) has preliminary data suggesting that low levels of IgA constitute a predisposing factor to the development of allergies in children. Also the fast decrease of IgE shown during the teens may provide an explanation for the long observed phenomenon that childhood allergies can be "outgrown". Floyd J. Malveaux (AI 05385, Johns Hopkins University) has shown that the total number of IgE receptors per basophil is closely correlated with the level of circulating IgE. This relationship implies a genetic association between serum IgE and the number of basophil IgE receptors or modulation of the number of receptors by circulating IgE. Charles W. Parker (AI 10405, Washington University) has been studying the IgE receptor on the rat basophilic leukemia cell line which he has been able to maintain in tissue culture. He has succeeded in solubilzing this receptor from the cell and purifying it to the point of almost complete homogeneity allowing possible design of soluble analogues of receptor which would compete with the receptor for IgE.

The interaction of IgE with mast cells and basophils and its importance in the allergic reaction has been amply demonstrated. Hans L. Spiegelberg (AI 10734, Scripps Clinic and Research Foundation) has recently found that IgE also binds to a subpopulation of bone marrow derived (B) lymphocytes. Peripherial blood contains approximately four percent IgE binding cells in contrast to tonsils and spleens which contain four to five times as many. These IgE binding B cells were also found in rats, usually at higher percentages than in man. The biologic role of the lymphocytes binding IgE is still unknown but studies are under way to determine the number of IgE binding B cells in patients with allergies and other disorders.

Another promising lead has been the determination by Dr. Wasserman that neutrophils, the primary cells responsible for defense against infection, are inhibited in their movement by allergic reactions. This

finding may help explain the frequent infections seen in some allergic individuals.

3. Asthma and the Other Allergic Diseases

Studies within this program have been concerned with investigations of the etiologic factors, pathogenetic mechanisms, diagnostic measures, and therapeutic approaches to the effective management of various allergic disorders. Among some of the diseases covered are asthma, allergic rhinitis ("hay fever"), hymenoptera sensitivity, and drug allergy.

Approximately 9 million Americans have asthma. Although asthma may have a number of different clinical presentations and the "asthma syndrome" may be comprised of several separate disease processes, the essence of asthma is reversible airway obstruction. This obstruction has three major elements: (1) contraction of bronchial smooth muscles, (2) increased or retained secretions of mucus, and (3) inflammation of the respiratory tract mucosa.

In a number of asthmatic patients, attacks can be provoked by exposure to specific allergens and many of the patients have other allergies such as allergic rhinitis. However, many asthmatics do not have a demonstrable allergy; and in these patients, a variety of infectious, nervous (adrenergic and cholinergic), genetic or inflammatory factors may play a primary, predisposing, or contributory role. The program on asthma is structured to investigate all the major parameters contributing to the totality of asthma. Areas under investigation include: natural history studies, allergens, bronchoprovocation challenge, animal models, pulmonary function testing, chemical mediators, cell mediators, adrenergic agonists and antagonists, parasympathomimetic agents, drug allergy, diagnostic methodology, and therapeutic agents.

The role of respiratory virus infections inducing asthma has been the effort of Charles Reed (AI 10404, University of Wisconsin). He has found that respiratory virus infections, particularly with rhinoviruses, may produce transient airway obstruction akin to asthma, and that the leukocytes from patients with rhinovirus illness have an impaired inhibition of lysosomal enzyme release from neutrophils. This observation has been extended to influenza virus which has been shown to induce changes in intracellular levels of cAMP. These findings may offer promising leads for utilizing changes in peripheral blood leukocytes to monitor bronchial responses.

For the past several years, inhalation challenge techniques have been refined to study the induction of asthma attacks. This methodology is now being employed by a number of grantees for a variety of investigative purposes: to corroborate a diagnosis of asthma, to ascertain the constituents of the chemical, cellular, and physiologic events in the ontogeny of the asthmatic response, and to evaluate pharmacologic agents in the treatment of patients. These bronchoprovocation studies have been conducted in asthmatic patients by Roy Patterson (AI 11759, Northwestern University) with inhaled antigens, such as ragweed antigen E, and with methacholine. He has found

evidence for a "seasonal priming" effect in that asthmatic subjects appear to be more sensitive to inhaled antigen when they are tested after a period of natural environmental exposure than before exposure. Attempts have been made, although thus far unsuccessfully, to correlate pulmonary function findings with the results of immunotherapy. If inhalation challenge responses were indeed prognostic in determining the therapeutic effects of immunotherapy, then obvious and immediate clinical and investigative benefit would ensue.

A colony of asthmatic monkeys has been developed by Dr. Patterson which appears to be the best animal model of asthma simulating the human disease condition. This antigen-induced asthma occurs only in selected monkeys which, like human asthmatics, have hyperreactive airways. Dr. Patterson has demonstrated that living antigen-reactive mast cells reside free in the bronchial lumens of these monkeys (as well as in dogs and man) and that these cells can transfer asthmatic type airway responsiveness to nonreactive animals. Moreover, a variety of chemical mediators of hypersensitivity, such as prostaglandins, histamine, and other agents postulated as being involved in the human airway response, have been tested in this animal model and shown to simulate the human asthmatic condition. In addition, the model provides a system of evaluating new therapeutic agents before they are tested in human subjects. The model also offers a unique opportunity to perform high risk repetitive studies under controlled conditions that could not be undertaken in humans.

Ragweed allergy is a disease which involves about 15 million Americans. For most individuals, it is a mild seasonal disease ("hay fever") characterized by eye irritation and nasal discharge; and its symptoms are usually relieved by antihistamines. For some patients, these symptoms, although not life-threatening, are a source of considerable morbidity. In others, ragweed is implicated in the causation of asthma.

In this allergic disease, there have been tremendous strides in our knowledge of its etiology, diagnosis, pathogenesis, treatment and prevention. As such, it may be considered a model of the progress we can expect in the future from a greater understanding of the various factors involved in all the allergic diseases.

Despite the fact that there are 100 different proteins in ragweed pollen, only five of them have been demonstrated to produce allergy in man. J. Donald Capra (AI 12796, University of Texas at Dallas) has determined the chemical structure including the total amino acid sequence of the smallest of these five proteins, termed Ra5. Within this year, he has completed the analytic structure of the next smallest molecule, Ra3 and determined that it is a 103 amino residue protein. He has found that the amino acid sequence of Ra3 is quite variable, with a number of amino acid substitutions occurring at different parts of the molecule. Moreover, these amino acid substitutions are associated with significant changes in biological activity as judged by histamine release assay.

Ra3 gathered in four separate sections of the country is subtly different--both chemically and biologically. This may be of considerable importance since the suppliers of pollen for use in human desensitization work

by allergists may vary their source. Thus, if an individual moves from one part of the country to the next, he may find that he is either more or less allergic to the "same" pollen. In addition, and more importantly, if an allergist should change his pollen supplier and/or if a supplier should obtain pollen from a different region of the country, the potency of the extract might be markedly different.

Gerald Gleich (AI 07047, Mayo Clinic) has successfully separated the two chains of antigen E, the major allergen of ragweed pollen. Biological activity of the individual chains was preserved, and studies are now underway to determine the amino acid sequence of each chain, including those portions of the chains which are determinants of the allergenicity of the molecule. If the ability to confer allergy resides in only a small portion of the molecule, then these studies could lead to a new and improved type of immunotherapy for ragweed pollen sensitivity. On the other hand, David Marsh (AI 09565, Johns Hopkins University) has focused his research on the highly basic proteins which are rapidly released from ragweed pollen grains, as contrasted with the acidic proteins, such as Antigen E, which are slowly released. Since allergenic reagents currently employed in immunotherapy contain a large proportion of acidic proteins, such as Antigen E, the addition of these rapidly released substances may lead to an improvement in immunotherapy.

A number of investigators, including Roy Patterson (AI 11403, Northwestern University) and Philip Norman (AI 04866, Johns Hopkins University) have pursued the development of chemically-modified ragweed allergens, termed allergoids. A variety of chemical agents (formaldehyde, urea, glutaraldehyde, ethylene, glycol, etc.) have been employed, but the main allergen studied has been antigen E. The principal advantage of employing these altered allergens in immunoprophylaxis is the more rapid induction of blocking IgG antibodies with greater safety than the conventional materials now in use. Larger amounts of immunogenic material can be administered in a shorter period of time without the risk of generalized allergic reactions.

David B. Dickinson (AI 12879, University of Illinois) has been studying the ragweed plant itself, and the genetic determinants of allergen production. Pollen has been gathered from different geographic sections, and both high and low yielding genetic strains have been identified.

A number of investigators such as Rebecca Buckley (AI 12016, AI 14314, and AI 70830, Duke University) have utilized purified ragweed antigens, such as antigen E, which are supplied by the NIAID Allergenic Reagents Program. With these purified reagents, Dr. Buckley and Dr. Patterson have been able to demonstrate the synthesis of ragweed specific IgE from lymphocytes cultured in vitro, thus establishing a laboratory-based system for further in-depth studies of human allergic disease. With this system, a number of provocative studies are already underway by several other groups of investigators to determine, in quantifiable terms, the molecular and biological mechanisms and their interrelationships which are involved in the development, maintenance and recovery from human allergic diseases.

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Concomitant with and because of the extensive use of purified ragweed antigens, existing stocks of these antigens in our Allergenic Reagents Bank were being depleted. It became necessary for the Allergy and Clinical Immunology Branch to advertise for a new contract to produce these reagents for distribution to the scientific community. Te P. King (AI 82567, Rockefeller University) an acknowledged leader in the field of ragweed antigen chemistry, was selected as the contractor to produce these reagents: Ra3, Ra5, antigen K, and antigen E. This contract, which has just gotten underway, will ensure the maintenance of a supply of these highly purified and well-characterized allergens for the exciting studies which lie ahead in ragweed allergy.

For centuries, it has been known that the sting of some insects in the bee family, called hymenoptera, could induce an overwhelming and even fatal reaction in certain sensitive individuals. About two million Americans are believed to be sensitive to these stinging insects, but the number of fatalities each year although not precisely known, is believed to be less than 1000. For those individuals who develop an overwhelming reaction to a hymenoptera sting, an injection of epinephrine ("adrenaline") is life-saving. Since exposure to these insects is most apt to occur in areas where prompt medical assistance is often not available, there was an obvious need for the development of an effective preventive measure. The hymenoptera include honeybees, yellow-jackets, hornets, and wasps. Their venoms contain several vasoactive amines and other noxious simple chemicals which are responsible for the immediate irritation experienced by anyone who is stung by these insects. In addition, venom contains several proteins, and it is these proteins which are responsible for the induction of the hypersensitivity state and the subsequent development of anaphylaxis in very sensitive individuals.

Until recently, the only materials available for testing and treating patients allergic to insect stings were the so-called "whole body" extracts, prepared by grinding up entire insects. Through the collaborative efforts of Lawrence M. Lichtenstein (AI 08270, Johns Hopkins University) and Philip S. Norman (AI 10304, Johns Hopkins University) it was established that the "whole body" extracts available to practicing allergists contain little, if any, active venom protein, and that these extracts have virtually no utility in the diagnosis and treatment of insect sting allergy. Subsequently, these investigators obtained venom itself from stinging insects, and prepared solutions for clinical evaluation in hypersensitive patients. There was an excellent correlation of the results of venom skin testing with the clinical history of hymenoptera sensitivity. Moreover, when these same venom materials were administered in a series of injections in what is known as immunotherapy to over 200 sensitive patients, protection against serious reactions ensued in well over 95 percent of patients. This finding was confirmed by actual challenge sting with the offending insect. In addition, the same venom materials were successfully applied in the laboratory to the development of a RAST procedure, which can diagnose insect sting hypersensitivity through the detection of insect-specific IgE antibody in the blood of hypersensitive individuals.

4. Immunodeficiency

The program on immunodeficiency is concerned with the etiology, ontogeny, prevention and treatment of structural and functional deficiencies of the immune system. Both naturally occurring and acquired disease states are included. Ongoing studies have focused on the congenital absence, failure of development, or other disorders affecting thymus or bone marrow cellular elements; abnormalities in production, inhibition, or catabolism of immunoglobulin; deficiencies of specific complement components; and defective host defenses due to abnormalities of leukocytes. These naturally occurring or acquired defects of immunity provide a unique opportunity to expand the understanding of normal immune function. Only by its absence, do we see a role for its presence in the sequence of steps which make up the normal immune response. Although these diseases are rare, the information gained is relevant to the general field of immunology and to many health problems including infectious diseases, cancer, allergy, and arthritis.

Of interest are immunodeficient subjects with high serum IgE concentrations. Dr. Buckley has examined the function of accessory cells such as macraphages and polymorphonuclear white cells in these patients, as well as in allergic individuals. Abnormalities of both monocyte and polymorphonuclear chemotaxis were found with some frequency in both types of patients. Richard Hong (AI 14354, University of Wisconsin at Madison) is looking at the exaggerated IgE response in allergic patients in terms of a possible deficiency in T lymphocyte regulation.

The role of thymus epithelial cells in reconstituting certain immunodeficient patients has previously been reported. He is studying improved methods of culturing the thymus prior to transplant and methods of predicting, in vitro, potential benefit of such transplants to patients. Dr. Hong now reports his overall success rate with approximately 20 patients so treated is about 50 percent. Ralph J. Wedgwood (AI 07073, University of Washington) has demonstrated the beneficial effects of cell free fluid taken from cultures of thymus epithelium in reconstitution of certain immunodeficiencies. Robert A. Good (AI 11843, Sloan Kettering Institute for Cancer Research) has discovered that aplastic anemia is not one but several diseases. Depending on the nature of the anemia, he has been able to effect cures by bone marrow transplantation or immunosuppresive agents. Edward J. Moticka (AI 12191, University of Texas Health Science Center, Dallas) has studied the acquired immunologic incompetence which accompanies antigen induced hypergammaglobulinemia. This interference with reactivity, probably an important regulatory mechanism for the immune system, may be responsible for the decreased immune capacity observed in patients with multiple myeloma.

Hugh Fudenberg (AI 13484, Medical University of South Carolina) has also been studying immune deficiency diseases. He has described an unusual array of genetic markers on certain lymphocytes of a subset of patients with "common variable agammaglobulinemia" who develop a fatal kidney disease. He has also found that IgA deficiency and X-linked hyper-IgM syndrome are heterogeneous diseases. In the latter disease, there is an apparent association between the disease state and blood group O, the closest association yet found between a given blood group and an immunodeficiency

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disease. His work on cystic fibrosis has led to the discovery of a protein in the serum of not only patients but carriers of the disease. Analysis of this protein marker called CFP, has indicated that the basic defect in cystic fibrosis may be related to a defect in another serum protein, alpha-2-macroglobulin.

5. Immune Complex Disease

Immune complexes result from the binding of antigens by antibodies. Subsequently, components of the complement system also become involved. Such complexes, therefore, arise in the course of the normal immune response. Under some circumstances, however, these entities appear within lesions and indeed are necessary for their development. The pathogenetic mechanisms by which immune complexes cause tissue damage, particularly in the autoimmune diseases with which they are increasingly associated, are still poorly understood. One deficiency, the lack of sensitive, quantitative assays for all types of immune complexes is being remedied. Vincent Agnello (AI 70968, New England Medical Center Hospital) has developed two such assays, one for "rheumatoid factor," an anti-IgG antibody, and one for the C1q component of complement. An unexpected finding which should correct misdiagnoses of systemic lupus erythematosus (SLE) is that some rheumatoid factors cross react with nucleoprotein. A further technological advance, due to Douglas B. Cines (AI 05477, University of Pennsylvania) utilizes the radioimmunoassay to quantitate IgG and the C3 component of complement complexed to a cell surface. This technique also lends itself to estimations of affinity of antibody for an antigen (R.M. Rothberg, AI 07854, University of Chicago).

William P. Arend (AI 10875, University of Washington) has sought to determine what renders an immune complex damaging. He reports that, in rheumatoid arthritis, anti-IgG antibodies (rheumatoid factor) specific for the Fc portion reacted with IgG to produce innocuous complexes, whereas anti-IgG antibodies specific for the Fab portion produced injurious complexes.

SLE is an autoimmune disease in which anti-DNA antibodies play a prominent pathogenetic role. The cause for the appearance of the autoantibodies is being studied in a genetically defined mouse strain which develops an SLE-like syndrome spontaneously. It appears that tolerance of self is broken because of defective suppression (M. Eric Gershwin, AI 00193, University of California, Davis). Studies in this strain have also been carried out to investigate the possibility of inducing tolerance to DNA by treatment with appropriately constructed antigens. Emergence of disease activity has been prevented and now attention is directed to treatment of the active condition (Yves R. Borel, AI 13867, Boston Children's Hospital Medical Center, and Bernard D. Stollar, AI 14534, Tufts University).

Immune complexes causing inflammation and tissue damage, are presumed to be involved in graft rejection. On the other hand, evidence has been obtained that such complexes can modulate the response to the antigen contained in them and prolong the survival of the graft. Consequently, Joseph D. Feldman (AI 07104, Scripps Clinic and Research Foundation) and associates have undertaken the analysis of the circulating immune complexes that appear after graft implantation and their effect of the on the host's response.

6. Inflammation

The process of inflammation is mediated by cells and by humoral factors. This process is essential for host defense against foreign substances. However, in certain disease states, inflammation plays a major role in tissue damage. An understanding of what initiates and what modulates inflammation is, therefore, crucial to explaining and controlling these disease states. The noncellular mediators of inflammation can be divided into two general categories, those of small molecular weight and those of high molecular weight. The group of serum proteins derived from the complement system and the associated enzyme systems of coagulation, fibrinolysis, and kinin fall into the latter category.

The initiation of the complement casade by either the classic or alternate pathway makes a major contribution to the development of the inflammatory response. Once activated, complement components have functions including opsonization, immune adherence, chemotaxis, cell lysis, and anaphylatoxin generation. Derangements of complement have been implicated in causing or contributing to a wide range of disorders including cancer, kidney diseases, collagen vascular diseases, infectious diseases, and inherited diseases.

Understanding of the complement system depends on isolation and identification of its components. Through work done by our grantees, it is known that there are approximately 20 complement components. Paul A. Liberti (AI 12365, Thomas Jefferson University) has devised a new isolation precedure for C_1q which results in stable preparations retaining all native biological activities. He has used this procedure to measure the physical properties of C_1q in order to determine its most likely molecular structure, thereby enabling them to relate its structure to function. Irma Gigli (AI 13809, New York University) is doing similar studies on proteins which are involved in the initial activation of the complement system in order to analyze their amino acid composition and sequence.

Particular interest has been placed on studies of the properdin or alternate complement pathway. The alternate pathway was, until recently, thought to have specific recognition proteins which bound to and thereby identified foreign particles for destruction. Hans Muller-Eberhard (AI 07007, Scripps Clinic and Research Foundation) reports that the complement protein, C3, has recognition function in the so called alternate pathway. Antibody is not required for this reaction. A cell (fungal, bacterial, etc.) that is an activator of this pathway allows C3, from the plasma, to be deposited on its surface. The deposited C3 interacts with surface structures of activators such that a regulatory protein which ordinarily initiates inactivation of deposited C3, cannot reach it. As a consequence, the orderly assembly of complement enzymes procedes without interference by regulators and the cell is killed by the membrane attack complex.

These basic studies have been very rewarding because they are useful in explaining mechanisms for several clinical problems. Understanding of these mechanisms is important for possible prevention and treatment of these diseases. For example, K. Frank Austen (AI 07722, Robert

B. Brigham Hospital) reports that he has been able to work out the molecular structure and mechanisms of action of C3 nephritic factor, the abnormal protein present in patients with membranoproliferative glomerulonephritis. This protein causes the complement system to be fully activated in the serum of patients with various types of nephritis. This protein is a host antibody directed against protein of the alternate complement pathway and serves to stablize the interaction of the complement protein thereby circumventing all host regulatory mechanisms. This leads to virtually complete depletion of the complement system with resultant inflammatory reaction. He is currently working out a role of the alternate complement pathway in bullous pemphigoid, a disease in which the mast cell effector system also appears to be abnormal.

Other tantalizing clinical leads have come from basic research in complement. For example, Dr. Gigli states that recent studies have shown that the complement system may play an important role in the localization of myocardial infarction. Depletion of one of the components, C3, is accompanied by better outcome of animals in which experimental infarcts have been induced. Carlos Arroyave (AI 14443,University of Colorado Medical Center) has pointed out problems of adverse reactions to dyes used in diagnostic radiography. He has shown that these substances can produce activation of the complement system causing release of histamine from blood cells. He is now investigating the possibility of designing an assay in the test tube capable of predicting which patients will react adversely to these dyes. John P. Leddy (AI 12568, University of Rochester) has been able to correlate the inordinate white cell depression described after donation of white cells to the activation and consumption of the complement system. This white cell activation is coincident with the formation of some white cell depressing factor in blood passing through the nylon filter used during this donation procedure (leukapheresis). By determining the precise mechanisms for this problem it may in the future be eliminated.

The genetics of the complement system have been studied by various investigators. Harvey R. Colten (AI 12791, Boston Children's Hospital Medical Center) has been looking at congenital absences of complement protein in regard to increased susceptibility to infection, hereditary attacks of edema, and increased susceptibility to rheumatoid-like diseases. Chester A. Alper (AI 14157, Center for Blood Research) has found that the second complement component and factor B but not the fourth component are controlled by genes in the region near the HLA genes.

A second category, that of low molecular weight mediators of inflammation, was previously mentioned. A discussion of basophils (or mast cells) must accompany any discussion of these chemicals, because these are the cells which release them. The sequence of events leading to mediator secretion is as follows: sensitization takes place; IgE attaches to the surface of mast cells and basophils; on reexposure, the cell-bound antibodies are bridged by the allergen signaling release of the mediators from the cells.

Research in this area has been facilitated by several new advances: Herve Bazin (AI 12830, Catholic University of Louvain) through the development of a colony of rats with IgE secreting plasmacytomas, has made

this immunoglobulin readily available; and both Dr. Parker and Dr. Ishizaka have established rat basophilic leukemia cells in culture.

Both of the latter investigators are also studying basophil receptors. Dr. Ishizaka has been able to demonstrate histamine release from these cells via divalent anti-receptor antibody without participation of IgE. Also studying these receptors is Floyd J. Malveaux (AI 05385, Johns Hopkins University) who has noted the relationship between serum IgE and the number of basophil IgE receptors.

While Dr. Ishizaka has shown that mast cells can discharge allergic mediators without involvement of IgE, Lawrence M. Lichtenstein (AI 11334, Johns Hopkins University) has demonstrated release of mediators from other than mast cells. Namely polymorphonuclear (PMN) leukocytes contain and release slow reacting substance of anaphylaxis (SRS-A) and eosinophilic chemotactic factor (ECF).

Philip S. Askenase (AI 12211, Yale University) is studying so-called cutaneous basophil hypersensitivity (CBH). Traditional views of delayed hypersensitivity and immediate hypersensitivity as being separate patterns of immune reactivity may no longer hold. For example otherwise typical delayed reactions contain significant numbers of basophils. CHB can be transfered in animal models by either immune cells or serum. Similarly, the late IgE mediated reactions in man may be analogous to serum transfer of CBH.

Despite this clouding of hypersensitivity reactions, some discussion of what is known classically as the type IV hypersensitivity reaction--cellular hypersensitivity, mediated by T cells is needed. These T cells also function as cytotoxic effector cells, have a role in secreting mediators of delayed hypersensitivity, regulate immune responses as helper or suppressor cells and act as memory cells.

The first part of this discussion will be confined to studies of hypersensitivity relevant to clinical diseases. Contact dermatitis is a prototype for cell mediated immunity. Henry Claman (AI 12685, University of Colorado Medical Center) has used a mouse model to study the development of delayed hypersensitivity to dinitrofluorobenzene. This experimental model is important because the complete antigen made after contact sensitization is DNP-coupled-to-self, and thought of in this way, contact allergy can be considered a form of autoimmunity where at least part of the antigen is self. Vera Byers (AI 12947, University of California San Francisco) is studying poison oak and poison ivy, two of the main causes of workman's compensation in California. She is developing systems to study the effects of these chemicals on human lymphocytes in vitro, in an effort to produce a state of unresponsiveness. Henry C. Maguire (AI 13337, Hahnemann Medical College and Hospital of Philadelphia) is also studying contact dermatitis. He is trying to determine the mechanisms of an interesting clinical observation--the use of cyclophosphamide under particular conditions will increase the intensity of allergic contact dermatitis reactions. Fred S. Kantor (AI 06706, Yale University) is studying the opposite phenomenon--anergy. He has induced anergy

in an animal model and found the effect to be mediated by a subset of T lymphocytes which adhere to nylon-wool columns.

When sensitized T lymphocytes are cultured in the presence of specific antigen, they release a variety of nonspecific mediators, or lymphokines, that act on other lymphocytes and macrophages among other cells. Manfred Mayer (AI 12371, Johns Hopkins University) has been doing basic research to determine what lymphokines are. In particular he has looked at lymphotoxin which plays a role in certain cytotoxic reactions mediated by lymphocytes, mitogenic factor, lymphocyte activating factor which is an amplifier of immune and allergic processes, and hydrophobic peptide. Louis W. Heck (AI 05207, Robert Beck Brigham Hospital) has been studying migration inhibitor factor. He has shown that macrophages, pretreated with proteinase inhibitors demonstrated an augmented response to subthreshold doses of MIF.

Eosinophils are known to be involved in inflammatory processes, particularly those allergic in nature. For this reason, some mention should be made of research in this area. Gerald Gleich (AI 09728, Mayo Clinic) has localized the basic protein of the eosinophil to the core of the eosinophil granule. The clinical relevance of this finding is, as yet, unclear. Thomas T. Hubscher (AI 15001, Georgetown University) has discovered that eosinophils possess various chemicals and enzymes that theoretically are capable of counteracting the harmful substances that lead to disease. Thus, eosinophils may serve to dampen the allergic response.

Macrophages which are differentiated blood monocytes differ from the other phagocytes in that they are long lived, are capable of continuing lysosomal enzyme synthesis, and may further differentiate into epithelioid cells which, in turn, may fuse to form multinucleated giant cells. The technology for these studies has been advanced by the work of Steven Douglas (AI 12478, University of Minnesota) who has developed a technique of freeze-fracture in conjunction with electron microscopy making it possible to study macrophage membranes at high resolution. In particular, he is interested in developing techniques for the study of the genetic regulation of their plasma membrane receptors for immunoglobulin. Victor Najjar (AI 09116, Tufts University) is studying a peptide called tuftsin which stimulates phagocytosis. He is looking at the possible curative ability of this compound in animals, especially those that have had their spleens removed. Barbara A. Nichols (AI 11639, University of California, San Francisco) is studying the role of macrophages in certain infections such as toxoplasmosis. She has studied factors which determine whether toxoplasma are phagocytosed by the macrophage. Through these studies she has determined that the motility of the toxoplasma is essential for penetration of new host cells.

GENETICS AND TRANSPLANTATION BIOLOGY PROGRAM

A. Scope

This Branch supports and manages research on the immunologic factors that determine the acceptance or rejection of grafts and on the genetic relationships that underlie them.

The increasing prevalence of degenerative disease and of trauma that may impair the function of particular organs sufficiently to be incapacitating or lifethreatening identifies the perfection of a successful transplantation methodology as one of the objectives of highest priority in current medical science. The technical surgical aspects have proved quite manageable but immunological rejection of the graft is the obstacle that has yet to be surmounted.

Research efforts supported by this Branch include:

1. Studies of strategies designed to minimize the likelihood of rejection by assuring the best possible antigenic match between donor and recipient.

2. Explorations of immunosuppressive regimes whose objective is to reduce the capacity of the host to reject the graft.

3. Studies of treatments of the graft that will reduce its ability to provoke a response.

Studies in animal models are supported that explore the genetic controls of the immune response, and, on a more practical level, test methodologies whose ultimate application is to man.

Genetically conditioned associations between antigenic characteristics and susceptibility to various diseases have recently been brought to light and also fall within the purview of this Branch.

Two important activities of the Branch are the management of the Kidney Transplant Histocompatibility Study which is now entering the followup and analysis stage and the management of the HLA Serum Bank, a national cell typing resource for transplantation as well as basic research studies. Stocks of human B lymphocytes are being accumulated and eventually will be made available to the scientific community for use in immunological studies and histocompatibility typing. A comparable bank of mouse typing alloantisera is also maintained by the Branch for distribution to investigators.

B. Awards and Support Levels

The Genetics and Transplantation Biology Branch supports research activities through individual grants, research career development awards, fellowships, training grants, and very importantly, in connection with its kidney transplant program and serum banks, through contracts.

The following shows the distribution of support for the activities of the Branch during FY 1978.

Genetics and Transplantation Biology
FY '78 Awards

Award Mechanism		Number	Amount[1]
Research Grants		46	$5,076,910
Career and Career Development Awards		8	266,571
	Subtotal	54	5,343,481
Fellowship Awards		11	137,461
Training Awards		1	58,405
	Subtotal	12	195,866
Contracts		43	2,242,593
	Total	109	$7,781,940

[1]Total costs, estimated using a 40% overhead for indirect costs.

The majority of these awards, approximately 85%, is concerned directly with transplantation biology and the remainder supports research on the genetics of the immune system and of disease associations. Approximately 40% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

C. Program Areas and Highlights

1. Genetics and Immunogenetics

The compatibility of a graft and its recipient is determined by their antigenic composition which is, in turn, determined by genetic factors. Attempts to identify these genetic factors in man began about 10 years ago. Considerable progress has been made through the application of statistics and population genetics. An obvious limitation, in view of the multiplicity of genes found to have been involved, is the unavailability of inbred genetically defined populations characterized by limited histoincompatibilities. This limitation is not nearly so severe in laboratory animals, although it is not totally absent. A most encouraging feature of work with animal models is their remarkably extensive homology at both the genetic and molecular levels with man. Studies on transplantation of tissues have been carried out with different objectives, at almost all levels of the animal kingdom. Among the mammals, guinea pigs, rabbits, and, most especially, mice have been preeminent. Indeed tumor transplantation in mice began about 75 years ago.

Histocompatibility in mice is determined by at least 30 and perhaps as many as 500 or even more different regions (loci) on various chromosomes, including the Y chromosome. The most important and most thoroughly studied complex of genes is on chromosome 17 and is called the H-2 complex.

Inbred strains of mice have been developed that are very similar in all aspects except for limited differences in the H2 system. The strains are defined by the antigens located on most cell types, including lymphocytes, and detected by specific antisera which cause lysis of these cells in the presence of complement. At this level, the H2 complex serves simply as a cataloguing system. However, mice with dissimiliar genetic compositions at the H2 complex are, as a rule, histoincompatible. Compatibility testing by grafting is cumbersome. Consequently, because rejection is predominantly a cellmediated immune reaction, in vitro tests involving the activation of lymphocytes have been devised. These include the mixed lymphocyte reaction (MLC) and its refinements in which the proliferation of host lymphocytes in the presence of donor cells is measured, and the cell-mediated lympholysis (CML) test in which host lymphocytes incubated with donor cells are examined for their ability to lyse the donor cells. The known lymphocyte activating determinants and effector cell stimulating determinants are encoded by genes in the vicinity of HLA of man and within H2 of mouse as well as in a separate region of chromosome 1.

Unfortunately, no more than in the case of the serologically determined antigens is there an absolute correlation between matching in terms of lymphocyte or effector cell stimulation and matching in terms of graft acceptance. The correlation is, however, high, indicating that the main graftrejection genes are linked within or near the major histocompatibility system. Nevertheless, the genes have not yet been identified. A major effort, supported by this Branch, therefore continues to be made by several investigators, including Donald C. Shreffler (AI 12734, Washington University), Jack H. Stimpfling (AI 06525, Columbus Hospital) and Henry I. Kohn (AI 14039, Harvard University) to breed new strains of mice, recombinants in the H2 region, in part to remedy this deficiency. Because of the participation of other loci in the determination of compatibility, such work cannot be totally successful. Studies on nonH2 loci are therefore also being supported. Donald W. Bailey (AI 13130, Jackson Laboratory) for example, has found two nonH2 loci, not identifiable serologically, in contrast to H2, whose effect on rejection of a graft is more than additive.

Another function of genes in the major histocompatibility H2 system is the control of antibody production in response to immunization with polypeptides and proteins. This exceedingly complex function, involving permissive as well as suppressive activities, is being investigated at the genetic level in mice by Martin E. Dorf (AI 13419, Harvard University) and by M. Boris Rotman (AI 12626, Brown University), and in the chicken by Arne W. Nordskog (AI 12746, Iowa State University). Dr. Rotman has identified a new locus, the IrZ gene, which controls the response to soluble bacterial beta-galactosidase; surprisingly, it is in two forms in different strains, being either dominant or recessive. Chemically distinct structures such as polypeptides with even minor differences in composition or sequence stimulate the production of antibody under the control of separate genes. Indeed, the different immunogenic sites of myoglobin have been recognized to be independent products of 3 or 4 genes by Chella S. David (AI 14764, Mayo Foundation).

Other aspects of immune recognition are associated with the antigens identified with products of the H-2 region and the I subregions. These are principally concerned with T-cell dependent humoral responses. For example, James M. Forman (AI 13111, University of Texas at Dallas) has reported that, to become cytotoxic after challenge with donor cells bearing the synthetic trinitrophenyl group (TNP), the responding cells must be presented with this group on a foreign H-2 antigen, that is, they recognize the TNP-H2 complex, not TNP alone. A more careful dissection of the problem of presentation of antigen by macrophages was made by Harry G. Bluestein (AI 10931, University of California at San Diego) who found that the macrophages and responding lymphocytes need be syngeneic (have the same genetic composition) only for the induction of proliferation in the responding cells. Syngeny is not required for recognition and the induction of some other responses such as increased amino acid uptake. Moreover, it is the I-region antigen on the lymphocyte, not the macrophage, that is critical, suggesting some interaction between this I antigen and the receptor for the foreign antigen.

Because the H-2 region antigens can be isolated by use of the sera available against them, studies of their physicochemical properties, such as those that result in their anchorage in the cell membrane have been undertaken by Leroy E. Hood (AI 10781, California Institute of Technology) and Benjamin D. Schwartz (AI 13782, Washington University). These investigators and others, including Stanley G. Nathenson (AI 07289, Yeshiva University) are also determining their sequences.

To facilitate these and related studies, the Branch manages a program of acquisition and distribution of mouse H-2 typing reagents and alloantisera. During FY 1978, approximately 1.4 liters of mouse anti-H-2 and -Ia sera were distributed to 110 investigators.

2. Transplantation Biology

It is recognized that significant progress in transplantation awaits a clearer understanding of the function of the immune system at its cellular and molecular levels than is currently available. Nevertheless, research focused on manipulation of the host or of the donor tissue to improve graft survival continues to be encouraged and supported.

At the practical level, Nicholas L. Tilney (AI 12250, Harvard University) is studying the nature of the cells responsible for graft rejection. He has found that, in the rat, removal of T cells leads to indefinite graft survival. Indeed the essential cell for rejection is the cytotoxic cell. Selective destruction of T cells can be accomplished with anti-thymocyte serum. Using the cruder anti-lymphocyte serum to pretreat his animals, Anthony P. Monaco (AI 14551, New England Deaconess Hospital) has demonstrated that further improvement in graft survival can be achieved by injecting donor bone marrow cells 7 days after grafting. Platelets given in large doses or with adjuvant are also effective when given 21 days after the graft is transplanted.

David A. Blumenstock (AI 12270, Mary Imogene Bassett Hospital) is exploring an approach of great theoretical interest and significant practical

potential. His findings in beagles suggest that lethal total body irradiation induces a tolerance-susceptible state. As a result, an allogeneic graft may be implanted successfully without prolonged immunosuppression. Immune reconstitution of the host is accomplished with autologous bone marrow.

The survival of a tissue transplant depends not only on the activity of the host's immune system but also on the antigens presented. These vary from tissue to tissue, and can vary even within a tissue. For example, Samuel L. Kountz (AI 12854, State University of New York, Downstate Medical Center) and his associates have demonstrated the appearance of new antigens in regenerating rat liver that render it recognizable by autologous lymphocytes.

3. Human Histocompatibility and Transplantation

The control of immunological characteristics in man bears many similarities to that in the best studied animal, the mouse, described above. The genes that are predominantly associated with histocompatibility are located on the short arm of chromosome six. They are designated the major histocompatibility complex or HLA system. Serological typing of cells has revealed 3 loci: HLA-A, HLA-B and HLA-C, each bearing multiple alleles at relatively low individual frequency. These genes code for glycoproteins of an apparent molecular weight of 43,000 which are, in turn, associated with another glycoprotein, beta-2 microglobulin, of 12,000 molecular weight. Products of the A and B loci have extensive sequence homology, indicating that the genes arose by duplication. To what extent the polymorphism at the A, B and C loci represents true allelism or close linkage of initially reduplicated genes is not yet known. The completeness of the serologic cataloguing is, in addition, thrown into some doubt because the assay, which tests for the presence of complement-dependent cytolytic antibodies, does not provide a complete screen. Thus, the presence of noncytotoxic antibodies with specificities for antigens associated with these loci has been demonstrated in some sera by absorption tests.

As in the case of the mouse, allogeneic stimulation of lymphocytes to proliferate or to differentiate into effector cytolytic or killer cells is dependent on loci separate from the HLA-A, B or C. The most important lymphocyte activation loci in the mouse, as stated above, map in the I region, between the serologically classified regions of H-2. In man, there are two such loci in the HLA region, the most prominent one being designated HLA-D. Also, as in the case of the mouse, closely linked to these genes are genes that code for antigens, primarily expressed on the surfaces of B cells, that may be identified serologically, these are called D-related or HLA-DR antigens.

Clearly, complete identity in antigenic constitution between donor and recipient results in the absence of immunological barriers to transplantation. Consequently, in the hope of minimizing rejection, extensive efforts are being made to match donors and recipients of grafts as completely as possible. Serological typing and matching at the HLA-A and B loci is done as a matter of routine whenever circumstances allow. Outcomes are most satisfactory when the donor and recipient are related. E. Donnall Thomas,

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a career award recipient (AI 02425) at the Fred Hutchinson Cancer Research Center of the University of Washington, has the most extensive experience in the area of bone-marrow transplantation. All tissue comes from related and matched donors. In cases of aplastic anemia, 50% survival has been attained, the longest survivor being 7 years after transplantation. If prior sensitization by transfusion is avoided as it has in the past year, rejection is not seen, and 17 of 18 patients are alive with successful grafts. Comparable though less dramatic results have been obtained with patients suffering from acute leukemia. Considerable efforts are underway to extend the scope and efficacy of matching by including typing at the HLA-D locus and its vicinity. Incompatibility at this locus is established in the MLC test and its refinements, in which the stimulation of proliferation of host lymphocytes by donor lymphocytes is assayed, as demonstrated by Fritz H. Bach (AI 08439, University of Wisconsin). This is a relatively cumbersome and time consuming test, even if the responding lymphocytes are primed (PLT and pPLT). In the mouse, the major lymphocyte-activation determinants map at or very close to genes that code for B cell antigens, and the same is true in man, where the DR antigens are closely linked with the D locus. For this reason, several investigators including Jean Dausett (AI 09293, University of Paris), Rene J. Duquesnoy (AI 12507, Milwaukee Blood Center) and Roy L. Walford (AI 10088, University of California at Los Angeles) have focused their attention on serologic typing of B lymphocytes. On the other hand, Soldano Ferrone (AI 13154) and Ralph A. Reisfeld (AI 10180) at the Scripps Clinic and Research Foundation are attempting to obtain more precisely defined antisera of very restricted specificity by extracting DR antigens from cell surfaces and immunizing animals with them (xenoantisera). This work, however, is still in the developmental stages.

Experience with kidney transplantation reveals, as with bone marrow transplantation described above, that although matching at the limited number of loci currently accessible is useful, especially when donor and recipient are related, it is by no means predictive of the fate of the graft. This is not surprising in view of the very large possible number of cell surface structures with antigenic potential (possibly as many as 500 or more in the mouse). In addition, the large number of loci and the polymorphism at each locus in man make the likelihood of truly satisfactory matching of unrelated recipients and donors an event of extremely low probability. Consequently, alternative approaches and methodologies to improve graft survival are being explored. Immunosuppression of the recipient is carried out routinely, usually by means of cortical steroids and cytotoxic chemicals. A more selective procedure employs anti-lymphocyte serum or globulin or, better yet, anti-thymocyte antibodies. This approach has been quite successful in certain animal species and has been proven useful when employed rationally and carefully in human transplantation. Hyung M. Lee (AI 12822, Virginia Commonwealth University) has attained, upon titration of recipients with anti-lymphocyte serum, graft survivals of up to 88%. The titration of the recipient with immunosuppressive agents is necessary because of the risks of infection attendant with their use. Consequently, measures of rejection are now being sought to serve as guides to the intensity of suppressive treatment. One herald of the onset of an acute rejection episode has been found by John Clancy, Jr. (AI 13186, University of Kansas) to be the appearance of neutrophilic monocytoid cells that synthesize DNA at highly elevated levels. This

occurs about 6 days before rejection is clinically apparent. At about the same time, T cells have been found to possess 3-4 fold increased levels of adenine deaminase, an enzyme of nucleic acid metabolism.

Efforts to improve the technical aspects of transplantation also are being encouraged and supported. For example, Giuseppe A. Andres (AI 10334, State University of New York at Buffalo) has found that the commonly used pulsatile flow perfusion apparatus may actually damage kidneys and precipitate hyperacute rejection. Alternative strategies to enhance graft and patient survival, based on studies on animal models, are under consideration such as the lethal irradiation followed by reconstitution with autologous bone marrow employed by David A. Blumenstock, which was mentioned previously. Still another potentially useful approach is being investigated by Lawrence A. Wilson (AI 11907, Louisiana State University Medical Center) who is attempting to modify the donor tissue so as to interfere with its recognition by the recipient. Here, this is accomplished by coating the tissue with antibodies specific for its antigens.

An integral component of the Branch's Program is the contractually-supported Kidney Transplant Histocompatibility Study which is a prospective study of the influence of a wide variety of parameters, including HLA compatibility, on the natural history of kidney transplants. Currently, 2,369 patients who received grafts at 43 participating hospitals in the U.S. and Canada from Jan. 1, 1974 to Dec. 31, 1976 are enrolled. Histocompatibility testing is based on NIH serum trays, as well as other reagents, is well standardized and subject to careful quality control. In addition, cells and sera from donors and recipients have been collected and frozen for further retrospective studies. Data on the recipients and the grafted kidneys will continue to be collected until Dec. 1978. Observations from 2 to 5 years after transplantation will, therefore, be available for detailed analysis which will be carried out in 1979. Preliminary analysis has revealed some salient points. HLA-A and B matching substantially improves graft survival when donor and recipient are related. At present, the data indicate that kidney graft survival at 2 years after transplantation reaches 86% if all 4 antigens are matched, 48% with a single antigen match, and 67% when no antigen matching was obtained. On the other hand, matching has relatively little effect when the donor and recipient are not related. The dominant influence on the success of transplantation between unrelated individuals is the age of the recipient, youth favoring prolonged transplant survival. Some factors with a relatively minor protective effect have also been identified, again principally when the donor is not related. Thus prior transfusion, nephrectomy with splenectomy and antilymphocyte globulin treatment and brevity of cold storage of the graft kidney appear to enhance graft survival. Whether these are truly significant will be determined only after the entire analysis has been performed.

To support these and related studies, the Branch manages the HLA Serum Bank which serves as a repository, distribution and quality control mechanism for human cell typing sera. Sera are acquired by purchase, contract or as gifts from scientists in the U.S. and overseas and are distributed for investigational purposes as well as for clinical applications. During the past

year, the Bank distributed 24 liters of antisera, including 30,500 histocompatibility typing trays, in 1700 shipments to a total of 304 U.S. and foreign investigators. These sera were used predominantly in pre-transplantation typing studies although they also have been employed for basic research studies in genetics, immunology and anthropology, among others. It is pertinent to mention that cell typing with these reagents has resulted in the discovery of some significant correlations between HLA type and certain diseases. Correlations involving myasthenia gravis, the degenerative neurological conditions, Alzheimer's and Jakob-Creutzfeldt syndromes, and multiple sclerosis especially in women have been identified recently by Roy L. Walford (AI 10088, University of California at Los Angeles). Even juvenile diabetes appears related to a particular HLA-D type. Causal relations are, of course, not yet available, but these associations may provide clues to disease mechanisms.

IMMUNOBIOLOGY AND IMMUNOCHEMISTRY BRANCH

A. Scope

This Branch is concerned with the biology and chemistry of the immune system and its products. The fundamental studies which it supports on the structure and function of the immune system are directed toward acquiring a complete understanding of immune response mechanisms at their basic cellular and molecular levels as they function in health and disease.

Activities in this broadly-based program area cross traditional disciplinary lines of biology and chemistry and encompass anatomic, physiologic, pharmacologic, and microbial biology and organic, physical and biological chemistry. Its scope includes studies of the origin, maturation, localization, and function of immunologically active cell populations, and the mechanisms involved in the induction, modulation, regulation, and expression of immune reactivity as well as physicochemical studies of antigens and their homologous antibodies and the mechanisms and kinetics of antigen-antibody reactions.

Research activities supported within this program area are grouped in the general programs for Immunobiology and Immunochemistry and in a Program of Institute Emphasis (PIE) for Lymphocyte Biology.

B. Awards and Support Levels

Relevant activities in immunobiology and immunochemistry are supported through various mechanisms including contracts, individual post-doctoral fellowships, institutional pre- and post-doctoral training grants, career and career development awards, as well as investigator-initiated research grants.

The following shows the distribution of support by award mechanism for the activities of the Branch during FY 1978.

Immunobiology and Immunochemistry Branch
FY '78 Awards

Award Mechanism		Number	Amount[1]
Research Grants		182	$17,503,866
Career and Career Development Awards		25	825,775
	Subtotal	207	18,329,641
Fellowship Awards		37	444,121
Training Awards		7	552,001
	Subtotal	44	996,122
Contracts		4	457,244
	Total	255	19,783,007

[1] Total costs, estimated using a 40% overhead for indirect costs.

The distribution of these awards by discipline is approximately 2/3 for immunobiology and 1/3 for immunochemistry. Approximately 30% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

Support for the Lymphocyte Biology PIE is included in the research grant category. During FY 1978, five program projects awards, at a total cost of $2,349,317, were made to support this activity.

C. Program Areas and Highlights

1. Immunobiology

This program is concerned with the processes leading to immunocyte differentiation, proliferation, and production of biologically-active substances which mediate immune reactions.

Research supported within this program includes studies on the origin, maturation, and localization of immunologically active cell populations, the interactions of lymphocyte subpopulations and their interrelationships with macrophages and other leukocytes, the cellular phenomena of antigen processing, immunologic tolerance and enhancement, and the mechanisms involved in the induction, modulation, and regulation of immune responses. The cellular mechanisms involved in the induction, maintenance, expression, and pathophysiology of delayed-type hypersensitive immune reactivity are also included in this category. Also relevant are studies of the lymphokines and other lymphocyte substances which activate macrophages and have pharmacologic effects on lymphocyte and other leukocytic cell functions such as chemotaxis, phagocytosis, blastogenesis, cytotoxicity, and microbial resistance.

By far the greatest activity in immunological research is directed toward the elucidation of the development, function and interactions of the cells of the immune system, the lymphocytes and the macrophages.

The response of an animal to the presence of a foreign substance, an antigen, in its internal environment is carried out by the lymphocytes which (1) recognize the substance as being foreign, (2) respond by a series of steps, including proliferation and differentiation, and (3) cause the removal of the foreign substance either via antibodies that they secrete or by direct attack. They are assisted in the recognition phase, the effector phase, and perhaps also in the intermediate phases, by large phagocytic cells called macrophages. These cells participate in the presentation of the antigenic material to the lymphocytes, secrete soluble products that regulate their activity, and assist in the removal of the antigenic material by ingesting and digesting it.

Lymphocytes may be segregated into two major classes according to their function, antigenic markers on their surfaces, and pathways of development, and also further into subclasses. Thus B cells have easily identified

immunoglobulins on their membranes and differentiate into antibody secreting cells. They originate in fetal liver and adult bone marrow. Alexander R. Lawton (AI 11502, University of Alabama at Birmingham) has succeeded in identifying and isolating a B cell precursor in man and mouse. This large cell lacks surface Ig but does synthesize it and represents the first recognizable stage in B cell development. It also serves as a landmark in the classification of deficiencies in antibody production. For example, sex-linked agammaglobulinemia represents a failure of differentiation beyond this pre-B cell stage, whereas in other types of agammaglobulinemia different blocks exist. The identification of this cell in mice presents the opportunity for experimental analysis of the genetic and environmental factors that control its development and differentiation.

These questions are also under active study at the Sloan-Kettering Institute for Cancer Research. Joan Abbott (AI 13759) is examining the differentiation of B, as well as of T, cells by means of the appearance or loss of membrane markers. These steps are rapidly inducible and do not require cell division. Paul W. Kincade (AI 12741) has facilitated the more precise dissection of these events by adapting a simple procedure to grow colonies of B cells from single ancestor cells (cloning). B cells, in the process of developing into antibody secreting cells, become committed to a particular isotype. Samuel Strober (AI 10293, Stanford University), on the basis of work with rats, has concluded that: (1) Commitment to the secretion of a given immunoglobulin does not require its prior expression on the surface. Thus cells with surface IgM can give rise to cells secreting IgM or IgG. (2) Cells possessing primarily IgD on their surface are memory cells destined to produce IgG, whereas cells with IgM and IgD are capable of producing IgM and IgG. (3) Cells with IgG on their surface can produce only IgG and suppress the IgM response to IgM and IgD bearing cells.

Many of these advances in understanding of cell properties depend on the recently developed fluorescence-activated cell sorter. This instrument has also been applied to the separation of T cell subclasses. T cells had previously been classified into three major subclasses according to their surface properties and distinct functions had been assigned to them. In the past year, Irving Goldschneider (AI 09649, University of Connecticut Health Center) has isolated, as discrete entities on the basis of surface markers, progenitors and precursors of lymphocytes. He has demonstrated that thymosin, a thymic hormone, is needed to stimulate the differentiation of T cells in the bone marrow. He has also associated a particular enzyme, terminal deoxynucleotidyl transferase (TdT), with lymphocyte development by showing an apparent precursor-product relation between cells in the thymus that possess and cells that lack it (AI 14743). John Crawford, a post-doctoral fellow (AI 05572) at the same institution, is exploring the effects of age on the functional capacities of lymphocytes and has already found changes in the distribution of the various populations with age.

Thymosin is not the only soluble regulator of lymphocyte development. For example, Margrit P. Scheid (AI 13374, Sloan Kettering Institute for Cancer Research) has found that the final step in the differentiation of B cells is initiated by cGMP and inhibited by cAMP and by the polypeptide hormone thymopoietin.

On the basis of work with T cell precursors and thymus extracts, J.J. Cohn (AI 11661, University of Colorado) has developed the interesting concept of a thymus-bone marrow feed-back loop. He believes that the thymus "senses" the quantity of T cell precursors that it contains. If they are insufficient, the thymus releases hormones which stimulate the marrow to increase the production of T cell precursors, which in turn migrate to the thymus.

B cells and T cells form a complex immunoregulatory network. The ability of B cells to produce a humoral response to challenge by certain antigens is critically dependent on the assistance of a subclass of T cells called helpers. Another subclass of T cells, on the other hand, has as its function the suppression of the response by B and other T cells. These activities, all set in motion by antigen, are controlled principally by genes located in the major histocompatibility complex, as is the interaction of the third cell type, the macrophage, with the lymphocyte. The genetic controls are under very active investigation by numerous laboratories. No clear pattern has emerged as yet. It does appear that: (1) Participation of macrophages and T cells is not required under all circumstances. Thus, there exists a class of antigens capable of stimulating B cells directly, the T independent antigens. (2) Response or failure of response to a particular foreign antigen is genetically determined. Absence of response may be the result of preferential activation of suppressor cells. (3) The functions of the macrophage are not all clearly indispensable. Its role in maintaining the viability of lymphocytes may be replaced by 2-mercaptoethanol. When the antigen is cellular, a sufficient cell density renders the macrophage unnecessary. The genetic restriction of the interaction of antigen-bearing macrophages with lymphocytes applies only to secondary, not to primary reactions. The restriction consists of the requirement that, for a secondary response, antigen be presented by a macrophage with the same surface markers as in the primary immunization. Under those circumstances, helper T cells are stimulated preferentially, whereas if the antigen is presented on other types of macrophages, the target population of lymphocytes tends to be the suppressor cells, as demonstrated by Carl W. Pierce (AI 13915, Jewish Hospital of St. Louis).

The manner in which antigen is presented is not well understood. David W. Thomas (AI 14226, Jewish Hospital of St. Louis) has obtained evidence that antigenic material initially bound to the macrophage surface is first occluded but becomes reexposed on contact with a T cell.

The problem of antigen-presentation is also being approached through studies on synthetic models. For example, Stephen C. Kinsky (AI 13414, Washington University), in pilot experiments with antigens incorporated into liposomes, has found that immunogenicity is markedly dependent on the lipid composition of the liposome.

Signal transmission to the B cell is also exceedingly complex. According to John W. Kappler (AI 11558, University of Rochester), the stimulation of a T cell by the proper antigen-macrophage combination appears to result in the release of an antigen-specific mediator which initiates the B-

cell response. A second type of T-cell then interacts with the B cell via the antigen and a surface marker to stimulate the differentiation of the B cell.

Because of the variety and complexity of the cell-cell interactions and because of the need to understand the intrinsic behavior of lymphocytes, cell culture techniques are being applied now in immunobiology. Although normal lymphocytes may be induced to undergo proliferation in vitro, they do not continue to divide and survive only for a limited time. However, "immortality" may be conferred on them by infection with certain viruses, by malignant transformation, or by fusion with cancerous cells such as those of myeloma. In the last instance, the products are called "hybridomas". The objective of such manipulations is the retention of a well-differentiated property by the continuously dividing cells, for example the production of specific homogeneous antibody, as demonstrated by Vincent R. Zurawski, a post-doctoral fellow (AI 05338) at the Massachusetts General Hospital.

Cell hybridization techniques have had one other highly informative application in immunology. Cell lines formed by the fusion of, for example, mouse and human cells suffer a progressive loss of chromosomes, principally the human ones. The remaining chromosomes are functional, resulting in the synthesis of proteins, including those expressed as cell-surface antigens. Because it is possible to identify which chromosome has been retained, genes for particular proteins can be localized on specific chromosomes and even on particular portions of chromosomes. In addition, the human antigens expressed represent a much narrowed spectrum. Indeed, individual human antigens can be isolated from such hybrid cells as reported by Barbara B. Knowles, a career development award recipient (AI 00053) at the Wistar Institute. This technique is therefore a very useful supplement to classical genetic analysis of immune marker distributions.

2. Immunochemistry

Research grouped in this program category centers on the defined molecular components of the immune system. These include the immunoglobulins that appear in the circulation, in secretions or anchored on lymphocyte membranes; the antigens both natural and synthetic; cell surface components related to immunologic activity; and subsurface cytoskeletal and contractile assemblies believed to play a role in cellular activation. Also within the purview of this program is the molecular biology of the immune response, that is the detailed structure of the immunoglobulin genes and the processes that lead to their expression. The newly-developing field of immunopharmacology, whose concerns are the identification or synthesis and characterization of chemical regulators of immune function and the elucidation of their mechanisms of action is, in addition, included in this program area.

The study of the structure of immunoglobulins has reached a mature state. Thus, over 700 sequences have been catalogued on the National Institutes of Health PROPHET computer system and have been used to predict the structural and functional roles of individual amino acids. This effort is supported by a contract to Elvin A. Kabat (AI 42147, Columbia University) and a career

development award to Tai T. Wu (AI 70497, Northwestern University).

Currently the most interesting targets of the investigations of protein sequences are the cell surface antigens, the products of the major histocompatibility system genes. As their name suggests, those genes are responsible for cell surface characteristics that determine the success of grafting. A clear understanding of the chemical nature of their products and the manner in which they interact with each other and with other elements of the cell is prerequisite to successful transplantation. Because of their very limited availability, progress in this area depends on the development of highly efficient procedures for the separation of the cleavage products obtained during the sequencing operations and of highly sensitive techniques for their detection. High pressure liquid chromatography and various other methods, including the use of electron capture technology and radiolabels, are being explored by J. Claude Bennett, (AI 09153, University of Alabama at Birmingham), Edgar Haber (AI 04967, Massachusetts General Hospital), J. Donald Capra (AI 12127, University of Texas at Dallas), Gerald M. Edelman (AI 11378, Rockefeller University), and Ellen S. Vitteta (AI 12789, University of Texas at Dallas). Results obtained so far by Dr. Edelman indicate that the mouse H2 antigens consist of one heavy and one light (b_2 microglobulin) chain, associated noncovalently. The heavy chain is anchored in the membrane through a hydrophobic carboxyterminal end. The H2 K and H2 D gene products are apparently homologous, suggesting that the genes arose by duplication of a common ancestor. The behavior of the H2 region antigens in the cell surface is also under investigation. Robert Cone (AI 12577, Yale University) is studying their biodynamics and has found that the antigens specific for B cells show biphasic kinetics, suggesting that there are at least two distinct populations of B cells. Those specific for T cells turn over at rates that depend on cellcell contact. S. Jonathan Singer (AI 06659, University of California at San Diego) has obtained evidence that the H2 antigens cocap, aggregating at a pole of the cell, with antigens. As a result, he has formulated a theory to account for the participation of the H2 antigens in the recognition of and response to certain antigens.

The definitive determination of structure is by xray crystallography. Its applicaton to immunoglobulins has proved quite difficult because of the flexibility of the hinge region which results in excessive looseness in the positioning of the Fc fragment. However, progress does continue to be made, principally, on the homogeneous myeloma proteins. Raghupathy Sarma (AI 10726, State University of New York at Stony Brook) has developed innovative approaches, including the use of styrene to increase the rigidity of the immunoglobulin molecules in the crystal and to reduce the damage normally produced by the ionizing xrays. Roberto J. Poljak (AI 08202, Johns Hopkins University) has determined the structure of a Fab fragment, as well as of a Fabligand complex. He concludes that the binding site hypervariable regions can accommodate substantial sequence variation without major structural alterations, an essential property in view of the heterogeneity of antigen-specific antibodies. Oliver A. Roholt (AI 12100, New York State Department of Health) has reported another important advance. He has crystallized the Fab fragment of antibody induced by immunization, which therefore has a known specificity in contrast to the fortuitous ligand affinities of myeloma

proteins. The structure of this "natural" antibody should therefore become identified in the near future.

Studies on the antigenicity of various structures have, generally, one of two purposes. One objective is to identify the specific features of the structure which serve as the ligands to the antibody combining site and to deduce therefrom the essential elements of the structure of the site as pursued by M. Zouhair Atassi (AI 13181, Mayo Foundation) and Emanuel Margoliash (AI 12001, Northwestern University). Alternatively, variations in the sequence and structure of chemically defined antigens such as the polypeptides and their derivatives are used to probe the functional properties of the components of the immune system. This approach has been very fruitful for decades and continues to provide useful insights. Thus David Givol (AI 15200, Weizmann Institute) and Michael Sela (AI 11405, Weizmann Institute) have found that immune responses to two polypeptides differing only in the replacement of phenylalanine for tyrosine, or simply in the order of a pair of amino acids in a sequence, are under separate genetic control in the mouse. Synthetic antigens may also have use in medicine. It is frequently possible to identify the ligand for which myeloma proteins, products of B cell tumors, have an affinity. As the cells possess receptors with the same specificity, cytotoxic drugs might be conjugated with tumor-specific haptens to enhance their localization in the tumor as demonstrated by Fred Karush (AI 09492, University of Pennsylvania).

The initial step in the triggering of the immune system is the acquisition by cell surface receptors of the appropriate stimulus, under normal circumstances an antigen. The ultimate responses of the stimulated lymphocyte are proliferation and differentiation. Of the events connecting stimulus and response almost nothing is known. Progress is beginning to be made in the elucidation of what transpires at the surface itself. On the phenomenological level, Gerald M. Edelman (AI 11378, Rockefeller University) has suggested that noncovalent but highly cooperative interactions of the receptors with subsurface cytoskeletal elements, the microtubules and microfilaments, extend across the membrane. The distribution of these and of contractile elements such as actin, myosin, alpha actinin and others, during the course of the proliferative response is being investigated by several laboratories.

There is currently little investigation of intracellular processes that constitute the essential elements of the response of lymphocytes to stimulation. Some years ago much activity was evident in the area of cyclic nucleotides and some new insights are still being brought to light. Byron H. Waksman (AI 06455, Yale University), in a careful analysis of the action of certain lymphokines in relation to the cell cycle, has shown that a T-cell product, "inhibitor of DNA synthesis", acts by stimulating the synthesis of cyclic AMP, but only in the G_1 portion of the cell cycle, and not in immature lymphocytes. Conversely, "lymphocyte activating factor" acts by increasing cyclic GMP synthesis, also only in G_1. Janos M. Varga, a career development award recipient, (AI 00154) at Yale University is using cytochemical methods to study differences between cells that bind or do not bind antigen. He has demonstrated that antigen-binding lymphocytes contain more DNA and are, therefore, at a different stage of the cell cycle

Molecular cloning or recombinant DNA technology and the highly efficient DNA sequencing techniques are being applied very successfully to problems in immunology. Janet M. Stavnezer (AI 14617, Sloan-Kettering Institute for Cancer Research) is conducting studies directed toward the enumeration of genes coding for the variable regions of immunoglobulin (the antigen-specific combining sites). This effort should clarify the controversy whether the great variety of variable regions is encoded within the genes transmitted from generation to generation or arises by somatic mutation within the given individual. The topography of the separate genes coding for constant and variable regions is being studied to elucidate the mechanisms of their assembly to produce, ultimately, the single polypeptide chain for which they code. The mRNA which is produced from these genes and its processing are being examined by Randolph Wall (AI 13410, University of California at Los Angeles). In addition, controls of expression of the genes that operate at the post-transcriptional level are being studied by Ursula B. Storb (AI 10685, University of Washington). Within a short time these investigations, because they are directed at the molecular events within cells, promise to resolve some of the most difficult problems in basic immunology.

3. Program of Institute Emphasis for Lymphocyte Biology

This Program has as its objective the rapid and efficient expansion of knowledge of all aspects of the physiological activities of the various subpopulations of lymphocytes. To this end, it currently supports five program projects, each a multidisciplinary effort integrating the technical expertise of cell culturists, geneticists, cellular immunologists and immunochemists, directed by acknowledged leaders in the field.

The project employing the most fundamental and molecular approach is guided by Gerald M. Edelman (AI 11378, Rockefeller University). It focuses on the analysis of molecular assemblies and their interactions at the surface and within the cells of the immune system. It has been highly productive and was renewed this past year for another term of five years. Much useful information has emerged from studies with the plant protein Concanavalin A (Con A). This material mimics, in many ways, antigens in their effects on lymphocytes. It induces a marked redistribution of the cell surface structures to which it binds and appears, thereby, to modulate the response of the lymphocyte to other mitogenic stimuli. Studies with it have led to a search for the structures and molecules within the cell with which the Con A receptor must interact to produce its stimulatory or response modulating effect. To deepen the understanding of the action of Con A, it has been subjected to x-ray crystallographic analysis. Structural studies of cell-surface proteins are also under way. More purely biological investigations are also within the scope of this program project. Thus, the ontogeny of antigen-binding cells is being clarified. For example, their absolute number in fetuses appears to be subject to two sets of controls, one of which shows simple mendelian inheritance.

The principal objective of the program project directed by Jonathan W. Uhr (AI 11851, University of Texas at Dallas) is the chemical and functional characterization of cell surface molecules, most specifically the immunoglobulins. Dr. Uhr and his collaborators pioneered the investigation of the physiologic significance of IgD as it appears on B cell surfaces. Most recent results suggest that cells bearing IgM and IgD can give rise to both IgM and IgG responses, whereas cells bearing IgD only or IgG can give rise to only IgG responses. IgD also appears to enhance the susceptibility of T-dependent responses to induction of tolerance but plays no role in the case of the T-independent response. A remarkable result has also been obtained in studies on antigen-specific T cells. These can be prepared in highly enriched form by attachment to antigen-treated macrophages. It appears, however, that the T cells do not recognize the native antigen itself but require the mediation of macrophages. Among the other activities within this program project are studies on the immunogenicity of derivatized histocompatibility antigens and sequence analyses of I-region antigens. This program project has just undergone competitive review successfully and is expected to be renewed.

The program project led by Matthew D. Scharff (AI 10702, Yeshiva University) encompasses the biology, genetics and biochemistry of lymphoid cells. A major area of current emphasis is the production of hybrid cells consisting of lymphocytes or macrophages joined to myeloma cells. The purpose is to establish monoclonal cell lines from which highly specific antibodies may be harvested in purity and quantity, as well as to investigate narrowly defined biological properties. In addition, mutants of myeloma cells with altered immunoglobulins are being isolated in the hope of gaining insights into genetic control mechanisms. Similarly, somatic cell hybrids with mouse cells are being made to localize genes on particular chromosomes. Mutants of macrophages defective in phagocytosis are also under study. On the more purely chemical level, the glycosylation patterns of immunoglobulins and the sequences of various proteins coded by genes of the major histocompatibility complex are being determined.

Fritz H. Bach (AI 11576, University of Wisconsin) is carrying out research of an almost totally classical immunologic nature. The objective of his program project is the elucidation of the roles of various surface antigens in eliciting known effector and suppressor activities in lymphocytes. The roles of cell-cell interactions in these processes are also studied as are membrane properties and their alterations during the differentiation of T cells. Somatic cell hybridization is being performed to produce cell lines with single specific immunologic characteristics, such as serologic haplotype. This will permit a more precise definition of the role of such characteristics.

A new Lymphocyte Biology program project grant was awarded during this fiscal year to a group headed by Baruj Benacerraf at Harvard University (AI 14732). It is designed to support basic research in immunology and immunogenetics, to assist in maintaining the colony of inbred congenic, recombinant, and allotype congenic mice which serve as a local and national resource, and to develop a biochemical laboratory for the characterization of immunologically important macromolecules.

Most recently, an application for a program project directed by Carl W. Pierce (AI 15353, Jewish Hospital of St. Louis) successfully underwent review and is being considered for award during the next fiscal year. It encompasses a variety of activities centered on the investigation of mechanisms of control of the response of lymphocytes to activating signals.

This Program of Institute Emphasis represents a major investment of the Institute in the concept that rapid advances in the understanding of the behavior of lymphocytes can be effectively achieved through carefully organized efforts by large, highly interactive groups led by well-established and highly productive senior scientists. To date, there is every indication that the Program has been successful and productive and that it will continue so in the future.

MICROBIOLOGY AND INFECTIOUS DISEASES PROGRAM

N I A I D

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viruses, eventual development of means of control depends on successful cultivation of these, and probably other viruses in cell culture. Reduction in the incidence of infantile diarrhea, a major objective, if accompanied by a decrease of similar syndromes in older children and adults, would impact not only on health but also on the $150 million spent every year for over the counter remedies for diarrhea.

Other viruses transmitted by the enteric route, as well as via transfusion, are the three or more agents of human hepatitis, types A, B, and non-A, non-B. The current technology which accomplished so much in defining the epidemiology of types A and B hepatitis, and in greatly reducing the hazard of type B associated with transfusion or dialysis, is now being applied to non-A, non-B. None of the viruses has yet been grown in cell culture, and the chimpanzee, susceptible to all three, has become an indispensable indicator of infectivity. A promising experimental inactivated type B vaccine prepared by concentrating viral antigen from the blood of chronically infected individuals has proven to be non-infectious in the champanzee, and these animals still demonstrated protection when rechallenged after one year. Although the antibody responses in the initial human safety test phase have been marginal, the prospects for a type B vaccine are real and encouraging. The control of type A and of type(s) non-A, non-B through immunization is dependent on successful growth of these viruses in a culture system suitable for vaccine production.

Viral vaccines provide protection by inducing an immune response, usually measured as serum or secretory antibody, and, increasingly, as cell mediated immunity. In addition to protection, the immune response may also be responsible for much of the tissue injury which accompanies a virus infection. It is a matter of fundamental importance to define how viruses affect the immune system and how components of that system act alone or in concert in limiting the spread of virus, in removing infected cells, or allowing viral persistence, as well as in mediating immunopathologic manifestations of infection. The ability to intervene at the level of the immunological response without compromising the host's ability to recover would offer new options for control of viral infections.

As for bacterial infections, these account for 90% of infections treated in the hospital, treatment that costs approximately $3.6 billion annually. Although the availability of many potent antibiotics has revolutionized the treatment of these infections, bacteria, along with fungi, have a remarkable capacity to survive in the environment and to colonize the immunocompromised host. As with viruses, the understanding of basic life processes at both the cellular and subcellular level will offer new options for the treatment and prevention of bacterial infections. More is being learned of the genetic basis for virulence-associated factors, and of extra-chromosal elements (plasmids) which determine toxin production and antibiotic resistance. Bacterial surface structures have been related to properties of virulence, and certain of these antigens have been associated with invasiveness, resistance to phagocytosis, and colonization. The tools are at hand for intensified studies of the molecular structure and function of bacteria, rickettsia, and fungi. One result will be new or improved bacterial vaccines. For multiple types of pneumococci and groups A and C meningococci, the polysaccharide surface antigens already have been used to produce effective vaccines. Vaccines

for additional types of meningococci, for gonococci, for Group B streptococci, and for Hemophilus influenzae type B are in various stages of development. Effective use of the latter seemingly will depend on the discovery of methods to enhance the immune response of children under two years of age. The availability of more and better vaccines, the appropriate use of antibiotics, and new approaches to immunotherapy, coupled with accepted practices of environmental control in the community and in the hospital, should add further years to the lives of U.S. citizens.

Citizens of many other countries are not so fortunate. There is a gap of 30 years between life expectancies in the more developed countries and those in the least developed countries. Up to 40% of children in some developing countries die before the age of five years, usually directly or indirectly because of viral, bacterial, or parasitic infections. Many of these fatal infections, plus uncounted non-fatal illnesses, are attributable to the fact that two-thirds of the people in the world's poorer countries have no access to safe drinking water or waste disposal systems, and virtually no access to basic health care. Until these situations are alleviated, it may seem that immunization and other control procedures have little to offer. On the contrary, as exemplified by the erradication of small pox and the effective use of measles and meningococcus type A vaccines in Africa, immunization may be one of the few approaches that can be employed pending environmental control and the provision of adequate nutrition and housing. This reasoning led to the widespread use of BCG to prevent tuberculosis in countries such as India, and it is of interest that such usage is now being reevaluated.

Since most of the least developed countries are in the tropics, their citizens are at risk of a number of infections peculiar to the tropics, particularly parasitic diseases, as well as to the bacterial and viral infections common to the rest of the world. Parasitic diseases still affect or threaten more than a thousand million people in tropical countries. One of the most important of these is malaria, the control of which was once considered within reach. But the number of cases of malaria reported to WHO has more than doubled in the last five years. In some countries there has been a 30-to-40-fold increase in cases reported since 1968-70. Mosquitoe resistance to insectide has been reported in 62 countries; strains of parasites resistant to chloroquine have been reported from 20 countries. Just as bacteria survive by developing the capacity to inactivate antibiotics, mosquitoes and malarial parasites acquire resistance to the chemicals directed against them. New approaches are needed. It is most encouraging, therefore, that investigators studying malaria, as well as those concerned with other parasitic infections, have been able to bring the contemporary tools of genetics, immunology, and tissue culture to bear on these problems. The capacity of the mosquitoe to transmit malaria may be altered by using biologic methods to control vector competence. Successful cultivation of the asexual erythrocytic stages of Plasmodium falciparum in vitro has made possible the production of antigen for an experimental vaccine, and permitted studies of the acquisition of resistance by the parasite to drugs such as chloroquine. Another vaccine approach has utilized the sexual stage (gametes) of P. gallinaceum to immunize chickens. When mosquitoes feed on the chickens, they injest antibodies against gametes; the antibodies block further development of the parasite in the mosquitoe, and, thus, interupt further transmission. Most recently, a gamete vaccine for P. knowlesi

in monkeys has been shown to prevent transmission of both the sexual and asexual stages. Equally imaginative approaches can be applied to other major tropical diseases such as schistomiasis, filariasis and leprosy if talented investigators can be found to undertake them. On May 9, 1978, HEW Secretary Joseph A. Califano, Jr., pledged to the World Health Assembly that the U.S. will seek "to increase support for training, for both our own people and those from developing countries, who can work as research scientists...", and "will seek to increase support for international activities of our own governmental agencies whose competence should be more readily available to international health activities". The Microbiology and Infectious Diseases Program is prepared to fulfill this pledge.

The two international programs currently supported are the U.S.-Japan Cooperative Medical Science Program and the International Centers for Medical Research Program, both of which operate under the authority of PL 86-610, the International Health Research Act of 1960. There are plans to expand the former and to change the latter.

The U.S.-Japan Cooperative Medical Science Program has been operative since 1965. It provides the basis for collaborative research between scientists of the two countries on diseases of importance to Asia, although some are also indigenous to the United States and Japan. The seven diseases or disease categories include: cholera, environmental mutagenesis and carcinogenesis, leprosy, malnutrition, parasitic diseases, tuberculosis and viral diseases. An eighth category, hepatitis, will probably be formally added to the Program in 1979. This will be the second addition since the inception of the Program in 1965. The first was environmental mutagenesis and carcinogenesis in 1972.

The annual meeting of the U.S. Delegation and the Panel Chairmen was held at the State Department on June 20, 1978, to review research progress and to prepare for the joint meetings in Tokyo in July. In consonant with panel member rotation, the membership of four panels was changed. These included the Cholera, Malnutrition, Parasitic Diseases and Viral Diseases Panels.

A Hepatitis Symposium was convened in Tokyo on July 17, 18 and 19, 1978, three days prior to the Joint Committee Meeting, as a preliminary step in establishing a Hepatitis Panel. At the conclusion of the Symposium, the following areas of mutal interest were identified by scientists from both countries:

1. Seroepidemiological studies in areas of interest, including China, Korea and Southeast Asia.
2. Development and evaluation of vaccines and the exchange of reagents.
3. Antiviral therapy, particularly for chronic carriers.
4. Non A and Non B Hepatitis.
5. Biochemical and antigenic characterization of hepatitis virus components.

The Joint Panels on Environmental Mutagenesis and Carcinogenesis also met before the Joint Committee Meeting. They conducted a workshop on the Utility of Studies for Mutagenesis of Sister Chromatid Exchange at Yamanaka, Japan on July 16 and 17, and convened a conference in Tokyo on July 18 and 19, 1978. Review and revision of panel guidelines was initiated to reflect future

research goals and objectives.

The Joint Subcommittee on Program Review and Planning met in Honolulu on February 9 and 10, 1978, and again on July 19, 1978, in Tokyo, just prior to the Joint Committee meeting. The latter was held at the Ministry of Foreign Affairs in Tokyo on July 20 and 21, 1978. Major topics discussed and acted upon at this meeting included the following:

1. The formation of a new Hepatitis Panel was approved in principle. The Subcommittee on Program Review and Planning will consider guidelines for the proposed new panel at its meeting in Hawaii in February, 1979.

2. Revised guidelines for the Malnutrition and Viral Diseases Panels were considered and approved. The Malnutrition Panel deleted studies on malnutrition due to metabolic disorders and added studies on health consequences of different (and changing) dietary patterns and food habits. The Viral Diseases Panel expanded its guidelines to include studies on viral gastroenteritis.

3. The formal reviews of the Parasitic Diseases and Viral Diseases Programs which were initiated in 1977 were completed at this meeting. The Committee accepted the reports as written and recommended that these Programs be continued.

4. The formal review of the Joint Environmental Mutagenesis and Carcinogenesis Program which was initiated at this meeting will be completed at the next Joint Committee Meeting in 1979.

5. The Joint Panels on Cholera and Malnutrition will hold conferences at NIH in July, 1979, just prior to the Joint Committee Meeting. With the completion of the review of these panels in 1980, the cycle of program review will have been completed a second time.

The International Centers for Medical Research (ICMR) Program was established in 1960 to advance the status of the health sciences in the United States and, thereby, the health of the American people through cooperative endeavors with other countries in health research, and research training. An ICMR may be described as a discrete research organization sponsored by a United States professional school (medical and/or public health) which provides a stable base for research and training through the development of research centers overseas. Collectively, the ICMR units have served as a national resource in creating a pool of investigators knowledgeable in treating tropical diseases and in stimulating young investigators to seek careers in international or geographic biomedical research.

The current and final funding period of the four ICMR grants ends May 31, 1980, after an existence of 20 years. A new initiative, International Collaboration in Infectious Diseases Research (ICIDR) will supplant the present ICMR program and will provide an opportunity to expand certain research activities, to initiate new ones and to establish additional overseas linkages.

The four institutions participating in the ICMR Program and their respective

areas of research interest are as follows:

1. The Johns Hopkins University ICMR is planning to relocate its overseas site from Dacca, Bangladesh, to the Gorgas Memorial Laboratories, Balboa Heights, Canal Zone. An attempt to return to the Cholera Research Center in Calcutta, India, was aborted by the Indian Government. The transition from Dacca to Panama should be accomplished quickly, and it is anticipated that the two years remaining in the granting period will be highly productive. Field work on parameters of diarrheal disease will be conducted in a Panamanian hospital with laboratory work being done at Gorgas. Only one investigator, Dr. David Sack, will remain in Dacca to complete his tour of duty there.

During the last year, three studies have shown that sucrose containing electrolyte solutions are as effective for rehydration as those containing glucose, both in children and adults, and in rotavirus diarrhea as well as in cholera syndromes. Studies are in progress in Dacca on the local intestinal as well as the systemic immune response to cholera; these studies are being done in collaboration with Dr. Pierce in Baltimore and Dr. Jan Halmgren in Sweden.

2. Tulane University in New Orleans maintains an overseas ICMR unit in Cali, Colombia. During the past year, there were 25 active projects in the general areas of parasitology, entomology (vectors and vector biology) and microbiology. There was a strong interest in parasitic diseases represented by several projects in helminthology (human and animal filariasis, echinococcus, trematode parasites) and protozoology (malaria, tryanosomiasis, amebiasis). In the area of microbiology, there were studies in virology (Guillain-Barre Syndrome, lower respiratory tract infections), mycology (paracoccidiomycosis, other deep mycoses), and bacteriology (diarrheal diseases). A site visit is planned for September, 1978, since this ICMR has not been visited since 1974.

3. The University of Maryland ICMR overseas site in Lahore, Pakistan, was site visited April 23-26, 1978. The Center had not been visited since 1976. During the visit and from subsequent correspondence with university officials, it was learned that Dr. Richard Baker will return to the University of Maryland in June, 1979, after spending eleven successful and productive years directing research at the Pakistan Medical Research Center (PMRC) in Lahore. Upon his return, he will succeed Dean Dennis as the Program Director of the PMRC, and will continue his mosquito genetic studies at the university. Dr. William Woodward, Associate Professor in the Division of Infectious Diseases of the Department of Medicine, will replace Dr. Baker at the PMRC. Work at the Center continues on mosquito genetics and ecology. There has been a definite but gradual shift toward studies with Anopheles culicifacies and Anopheles stephansi, mosquitoes which are principal vectors of malaria in Pakistan and of the Pak-India subcontinent. Pilot field studies with genetically marked mosquitoes are in progress. The work has been expanded to include a virologist who is working with the geneticists to develop a strain of Culex tritaenorhynchus that will not transmit a local arbovirus. If time permits, the virological work will be expanded to include studies of Congo virus ecology and epidemiology in northern Pakistan. It was recommended by the site visitors that an epidemiologist be added to complement and to increase the depth of this excellent staff.

4. The University of California ICMR overseas site in Kuala Lumpur, Malaysia, was site visited from April 27 to May 1, 1978. Presentations were given by various investigators involved in arbovirology, parasitology, community health, and human genetics and hematology. In general, the quality of research was considered quite good, although the site visitors were concerned that some of the young, inexperienced investigators lacked appropriate preceptorship. However, the ICMR program appears to be truly integrated with its counterpart in Malaysia, the Institute of Medical Research (IMR). The Director of the IMR indicated that the association was mutually beneficial. Other institutions affiliated with the ICMR are the University of Malaya and the University of Kebangsan.

ICMR Evaluation: Presently, there are plans being formulated with 1% set-aside funds to have a qualified contractor, yet to be selected, perform an in-depth, critical review and evaluation of the ICMR program from its inception in 1960 to the present. Data to be sought should be useful in modifying or revising on-going programs and in providing constructive criteria for establishing new tropical disease initiatives.

International Collaboration in Infectious Disease Research is a new program initiative announced by the Microbiology and Infectious Diseases Program in the May 12, 1978 issue of the NIH Guide to Grants and Contracts. The program is divided into Part A, International Program Project Grants, and B, International Exploratory/Developmental Research Grants, to provide flexibility and to insure maximum opportunity for participation. The research emphasis of the new ICIDR program is infectious diseases and the immunology of these diseases. Special attention is given to the six diseases of the WHO Special Program for Research and Training in Tropical Diseases.

Part A, International Program Project Grants, embraces broadly based multidisciplinary research programs that have a well-defined central focus or objective, with major portions of the research conducted overseas with an acceptable foreign affiliate.

Part B, International Exploratory/Developmental Research Grant, encourages the individual investigator to develop a biomedical research program with an overseas affiliate, with most of the work being done in the host country.

U.S.-U.S.S.R. Health Cooperation. In addition to the above, NIAID is a participant in the U.S.-U.S.S.R. collaborative program in the field of influenza and acute respiratory diseases. During the four years the program has been in existence, efforts have been directed toward five problem areas related to influenza. Drs. Jordan and Galasso serve respectively, as U.S. coordinators for two of these areas: I. etiology and specific immunoprophylaxis; IV. chemointerferon prophylaxis and therapy.

CLINICAL STUDIES BRANCH

The Clinical Studies Branch serves as the Institute's focus for the development and processing of Investigational New Drug Applications. It provides and monitors closed clinical facilities for Institute volunteer studies and maintains liaison with the NIH Human Research Review Panel and the NIAID-Clinical Research Subpanel. It conducts, promotes and supports the development and evaluation of procedures for the diagnosis, prevention and treatment of infectious diseases, particularly those procedures leading to improved patient care.

Approximate Level of Support

Activity	Number	Amount
Research Grants	2	$118,266
Research Contracts	2	$1,297,622
Training Grants	0	0
Fellowships	0	0
Total	4	$1,415,888

Program Areas

Closed Volunteer Facilities

The Clinical Studies Branch currently supports one research contract with the University of Maryland. This contract is for the maintenance and support of a facility in which closed populations of volunteers are studied following administration of infectious, and therefore, potentially transmissible agents. During the past year, the contractor has evaluated seven different live bacterial or viral pools in a total of 13 studies. The investigators discovered that enteropathogenic E. coli strains, epidemiologically associated with outbreaks of infant diarrhea, that did not elaborate heat labile (LT) or heat stable (ST) enterotoxin and were non-invasive, nevertheless, had the potential to cause diarrhea in adults. From these strains a new enterotoxin has been discovered that appears distinct from ST and LT as defined by classic assays. They identified two El Tor Inaba cholera strains for use as challenge organisms in future vaccine efficacy studies, and characterized El Tor Inaba cholera in the volunteer model. Studies with A/Victoria/3/75ts 1A-2 (H3N2) vaccine virus showed that it was well-tolerated by volunteers, and that it was immunogenic and protective. Results from an A/Alaska study were similar for safety and immunogenicity; however, protective efficacy has not yet been tested with this strain. Virulences of recipient wild virus strains of A/Alaska and A/HK (H1N1) were determined. A rotavirus homologous re-challenge study demonstrated protection against homologous human rotavirus type 2 infection.

In the laboratory these workers have developed a sensitive enzyme-linked immunosorbent ELISA assay for cholera and E. coli serum antitoxin which will

now be adapted for detection of secretory antibody. They have succeeded in maintaining in tissue culture two human intestinal epitnelial cell lines which are currently being used for testing adhesion potential of various E. coli enteropathogens. Investigations of oral glucose electrolyte therapy in volunteers with cholera and E. coli diarrheas have been done, as well as studies comparing glucose electrolyte and sucrose electrolyte formulas in treatment of infant diarrhea due to various etiologies, including rotavirus infection. Glucose is slightly better than sucrose with a 95% as compared to 90% success rate irrespective of etiology.

Collaborative Clinical Trials With Antibiotic Therapy

The primary goal of this program is to sponsor collaborative clinical trials designed to improve care of patients with bacterial and mycotic infections. Approved antibiotics, or new drugs that have promise but lack the economic potential to justify testing by the pharmaceutical industry, are assessed for possible evaluation in patients with selected infections.

During FY 78 two scientific conferences were sponsored by the CSB for the purpose of promulgating scientific exchange of information and developing new initiatives for the program.

At the "Technical Workshop on Amphotericin B Methyl Ester (AME)", held February 21, 1978, program staff met with thirty representatives of the scientific community and pharmaceutical industry to review the current status of AME. The purity, stability, toxicity, and efficacy of AME were reviewed and compared to the licensed drug, Amphotericin B (AMB). The feasibility and applicability of using AME in controlled clinical studies for therapy of systemic mycotic infections were evaluated. It was concluded that cryptococcal meningitis, the cutaneous form of disseminated coccidioidomycosis and pulmonary histoplasmosis would be most suitable for clinical studies to determine efficacy and adverse reactions. Compared to Amphotericin B, AME has less renal toxicity, and it may have application in numerous clinical situations especially those where renal function is compromised. After this workshop, the CSB issued an RFP for the improved therapy of cryptococcal meningitis; a three year contract was awarded to the University of Alabama. The contract will consist of a 14 multi-institutional collaborative trial divided into three sequential studies. These studies are designed to 1) determine the most efficacious, minimum-duration treatment regimen utilizing AMB and 5-fluorocytosine (5FC), 2) determine toxicity and appropriate dosage of AME in patients having certain fungal infections other than cryptococcal meningitis and 3) compare, in a randomized trial AMB-5FC and AME-5FC for the therapy of cryptococcal meningitis.

The purpose of the "NIAID Symposium on the Impact of Infections on Medical Care in the U.S." held May 30-31, 1978 was to explore the nature and magnitude of infectious problems faced by the health care delivery system, to examine current methods of prevention, diagnosis and management, and to evaluate the requirements for future research, training and manpower which might alleviate the serious impact of infectious disease in hospital and community settings. Four areas for possible future research support were identified: 1) the epidemiology of hospital and community infections, 2) evaluation of whether

clinical microbiology services can improve management of infections, 3) clinical trials with antimicrobials for selected infections, 4) studies of the factors leading to the inappropriate use of antimicrobials by practitioners.

It is apparent that area #3 is directly related and most relevant to the mission of the CSB. New initiatives shall be developed that focus on improved drug therapy for selected infections. Through cooperation and collaboration with other components of MIDP, the CSB can contribute to new research activities that will alleviate problems defined in the remaining three areas.

Rapid Viral Diagnosis

The long-term goal of this program is to provide the physician in a primary care facility with methodologies and techniques so that a specific viral diagnosis can be made in time to aid in the management of the patient. Rapid diagnosis of a specific viral disease could result in reduced use of antibiotics, better monitoring and reduction of nosocomial infections and allow the use of effective antivirals when they become available.

A workshop was held on January 12 and 13, 1978 on "New and Useful Methods in Viral Diagnosis." A summary of it will be published in the September issue of the Journal of Infectious Diseases as part of the NIH News.

One of the most valuable aspects of the workshop was the gathering together of administrators, investigators and representatives from commercial firms who then discussed common problems, goals and accomplishments. Close collaboration with North American and European groups is continuing so that duplication of effort will not occur. A research program is being developed in a sequential fashion. Two newly awarded grants on rapid diagnosis of respiratory virus and arbovirus infections have been assigned to it this year. An RFP to exploit the ELISA assay for the diagnosis of selected respiratory virus infections is being developed.

Nutrition, Infection And Immunity

The goal of this program is to promote research on the interaction of malnutrition and infections in American hospitals and in overseas populations. Because we regard the malnourished patient at high risk for infection, the goal is consistent with the overall mission of the CSB which is to encourage clinical and basic research leading to improved patient care.

During FY 78, Dr. Edelman served on the NIH Nutrition Coordinating Committee (NCC). Through this committee and in an effort to stimulate research in nutrition, he helped design and initiate several new nutrition research initiatives scheduled to begin this year or in FY 1979. These new initiatives include the establishment of clinical nutrition research units with NIAMDD as lead agency, a program announcement on nutrition sponsored by the U.S. - Japan Malnutrition Panel, and a draft program announcement on obesity in collaboration with several other institutes. Infection and immunity research is represented in each of these new nutrition programs. Dr. Edelman chaired several working committees in the NCC: an ad hoc study section to evaluate a

conference travel grant submitted by the Eleventh International Congress of Nutrition in Brazil, a pan-NIH committee to write the nutrition policy statement for the NIH, and a program planning committee for a NCC-sponsored national conference designed to develop a consensus on the use of total parenteral nutrition. Dr. Edelman initiated and helped draft an agenda for an International Symposium on Nutrition and Immunity, tentatively scheduled for FY 79 under pan-NIH sponsorship if approved by the Institutes concerned.

Staff of the CSB continued their collaboration with the Diet, Nutrition and Cancer program of the National Cancer Institute by assessing the role of hyperalimentation and improved nutrition on infections and the immune response in selected cancer patients. In overseas oriented nutrition research, Dr. Edelman delivered a paper entitled "Malnutrition and Leprosy" at the Indo-American Conference on Leprosy Research in October 1977 held in Agra, India, which was sponsored by the Indian Council of Medical Research. During this trip he helped design several collaborative research projects on nutrition and enteric (rotavirus) infections with Indian investigators in Calcutta and Hyderabad and with Dr. Yolken of the LID, NIAID. In a visit to Thailand he served as a technical consultant to the Anemia and Malnutrition Research Center, Chiangmai University, Chiangmai, Thailand. Together with Dr. George Curlin of NIAID, Dr. Edelman initiated discussions about a new collaborative overseas program on Infant Diarrhea, Nutrition and Immunity with the Agency for International Development, U.S. State Department.

Other Activities

The responsibility for collecting and reviewing the necessary documentation required to file and maintain Investigational New Drug Applications (INDAs) with the Food and Drug Administration (FDA) for the Microbiology and Infectious Diseases Program resides in this Branch. Currently 20 active INDAs are filed with the Bureau of Biologics, FDA, and three with the Bureau of Drugs, FDA. During the past year new INDAs were filed for the study of "Escherichia coli Somatic Pilus Vaccine," "Gonococcal Protein Vaccines" and "Inactivated Influenza Virus Vaccine (A/USSR)". INDAs will be filed for "Group B Streptococcal Antigens", "Amphotericin B Methyl Ester" and "Acycloguanosine" later this year. Staff support is provided for the NIAID-Clinical Research Subpanel (CRS) which reviews both intramural and contract-supported clinical studies. Staff assures that all MIDP studies involving humans are reviewed by the CRS initially and every three years thereafter, and by the NIAID-Clinical Director in intervening years.

As an ex-officio voting member of the National Commission on Digestive Diseases, Dr. Edelman participated in numerous public hearings of the Commission, helped organize a Commission-sponsored symposium on nutrition and digestive diseases, and served as a member of 3 subcommittees: research, prevention, and editorial, all directed toward developing a long-range plan in digestive diseases to be contained in a report to Congress due October, 1978. Dr. Edelman also served as NIAID representative to the Congressionally mandated Digestive Disease Coordinating Committee of the Federal Government.

Dr. Horton served as liaison member of the NIH Coordinating Committee on

Cystic Fibrosis. Specific areas for expanding NIAID's research in cystic fibrosis were identified. A trans-NIH research program announcement in cystic fibrosis was released in January 1978. A conference in cystic fibrosis research, jointly sponsored by Cystic Fibrosis Foundation, NIAID and NIAMDD has been scheduled for February, 1979.

EPIDEMIOLOGY AND BIOMETRY BRANCH

The Epidemiology and Biometry Branch plans, conducts, and coordinates a program of epidemiological research into the origins, frequency of occurrence, and georgraphic distriubtion of infectious diseases in humans; plans, develops, and coordinates the Institute's program for mathematical and statistical studies of infectious diseases; analyzes data from various sources for trends in U.S. vital statistics in areas of interest to this Institute's programs; provides service and consultation in the application of advanced mathematical and statistical techniques to infectious disease research; collaborates in devising appropriate study designs and sample sizes for clinical intervention studies; and provides consultation on epidemiologic aspects of infectious diseases to physicians and scientists.

Approximate Level of Support

This is primarily a consultant Branch; it does not yet administer any grants or contracts.

Staff Changes

Two professionals were added to the staff this year, enabling the Branch to increase its involvement in Institute programs. William Blackwelder, Ph.D., was appointed biostatistician on January 1, 1978, and Ms. Jacqueline Smith was appointed computer specialist in March.

Consultative Services

The EBB has provided technical assistance in epidemiology and statistics to a number of other program areas during the past year. EBB staff assisted in analyzing data generated through contracts in the fields of antiviral chemotherapy for herpesvirus keratitis, hepatitis B virus epidemiology, and pneumococcal polysaccharide immunoprophylaxis for recurrent otitis medi in children. In addition, EBB staff assisted in reviewing the design and data management considerations of proposed clinical trials.

During the current year, the EBB engaged in a collaborative study of host factors in recurrent herpesvirus infection in the well-characterized population of Framingham. The histocompatability type is being determined in a carefully drawn 10% sample of the population which is under surveillance for recurrent herpes infections. Dr. Fred J. Payne is co-Principal Investigator in this project which is being conducted in conjunction with the Bureau of Biologics and the National Heart, Lung and Blood Institute.

Technical Assistance

Following the appointment of Ms. Smith and the establishment of access to NIH computer facilities, the EBB was able to actively participate in the design, management and analysis of data from the large-scale clinical trials of influenza vaccine conducted during the past spring. Thus, the Branch is now able to provide data processing and analysis support for a National program;

it is expected that it will expand its role in direct support of this and other similar Institute programs in the future.

Workshop

On March 2 and 3 the EBB sponsored a workshop to consider the advisability of including histocompatability phenotype among the variables in future epidemiologic studies of infectious diseases. Workshop participants represented the disciplines of infectious diseases, epidemiology, immunology, biostatistics, genetics and tissue typing in roughly equal proportion. The workshop recommended that histocompatability typing be combined with infectious disease studies with priority being given the following: 1) efficacy trials of vaccines with emphasis on those vaccines which are defined on a molecular basis, 2) prospective studies of specific infectious diseases in populations of families which are currently underway or in the process of being implemented, 3) instituting infectious disease studies in populations where histocompatibility phenotype has already been determined. There was an emphasis on the need to conduct carefully designed epidemiologic studies in this area which has often been characterized in the past by associations drawn from poorly controlled observations. The conclusions of the workshop are being published and will form the basis for a decision as to whether or not support for such studies will be programmed in the future.

Collaborative Studies

Analyses begun last year to utilize data from the National Center for Health Statistics, IMS America and the Health Care Financing Administration to obtain improved measures of the economic and social impacts on both infectious and allergic diseases have continued. The EBB has provided data to the Task Force on Asthma and Other Allergic Disorders of the IAIDP, NIAID, on the economic and social impacts of the allergic disorders utilizing the above sources. Work is currently underway to collate such information relative to the infectious diseases. EBB staff also has served on the Public Health Service Task Force on the Cost of Illness.

During the past year, the EBB was identified as the focal point for a contract for a large Participating Agency Service Agreement with the CDC in support of the conclusion of the Study on the Efficacy of Nosocomial Infection Control (SENIC). This large project on nosocomial infections is expected to identify problems for which specifically targeted research will expidite progress in this increasingly important area. The SENIC project is currently in the final phase of the Medical Record Review, and preliminary data suggest that this retrospective method for determining nosocomial infection rates represents a technological break through which will greatly facilitate research and improve control of these all too common infections.

DEVELOPMENT AND APPLICATIONS BRANCH

A prime mission of the NIAID is the control of infectious diseases. The objective of the Development and Applications Branch is to translate findings from basic research into methodologies that can effectively and rapidly be used in the clinic. By definition, the programs assigned to the Branch are those identified as high priority areas; these include: Influenza, Respiratory Disease, Hepatitis, Antiviral Substances, Bacterial Vaccines, and Enteric Diseases.

Approximate Level of Support

Activity	Number	Amount
Vaccine Evaluation Centers		
Research Contracts	5	$ 1,274,882
Influenza		
Research Contracts	15	2,267,935
Interagency Agreements	3	61,030
Research Grants	20	1,678,937
Fellowships	2	24,600
Respiratory Diseases		
Research Contracts	1	60,300
Interinstitute Agreements	1	43,474
Research Grants	3	229,800
Hepatitis		
Research Contracts	7	966,032
Interagency Agreements	2	131,175
Research Grants	6	655,447
Career Awards	2	75,305
Fellowships	1	13,200
Antiviral Substances		
Research Contracts	14	1,465,968
Research Grants	20	1,570,193
Career Awards	1	38,839
Fellowships	2	21,165
Training Grants	2	118,637
Bacterial Vaccines		
Research Contracts	10	1,305,337
Interagency Agreements	1	107,000
Research Grants	10	590,343

Approximate Level of Support (cont.)

Activity	Number	Amount
Bacterial Vaccines (cont.)		
Career Awards	1	$ 39,610
Fellowships	2	29,400
Training Grants	1	80,984
Enteric Diseases		
Research Contracts	7	802,460
Research Grants	19	1,319,981
Career Awards	1	39,528
Fellowships	3	37,600
Branch		
Research Contracts	59	8,142,914*
Interinstitute Agreements	1	43,474
Interagency Agreements	6	299,205**
Research Grants	78	6,054,701
Career Awards	5	193,282
Fellowships	10	125,965
Training Grants	3	199,621
Totals	162	$15,059,162

*includes $1,118,616 of National Influenza Immunization Program funds
**includes $ 37,530 of National Influenza Immunization Program funds

Program Summary

The past year has been one of intense activity for the DAB highlighted by the increased activity in the Influenza Program as a result of the appearance of a new strain (influenza A/USSR/77). The DAB utilized its contractors in conducting research, in collaboration with the Center for Disease Control and the Bureau of Biologics, to establish the appropriate vaccine dosage for the general public in the 1978-79 season. The Program also rapidly put into effect much needed field studies to evaluate the efficacy and side effects of amantadine/rimantadine. Other studies on attenuated and subunit vaccines, immunologic, epidemiologic and pathogenic characteristics are proceeding well, as are vaccine studies in the pediatric population.

To develop a stronger basic knowledge data base for respiratory syncytial virus and parainfluenza viruses, an announcement for research grant applications was released. It is hoped that increased emphasis on the viral causative agents of lower respiratory disease in pediatric populations will produce new insights into development of control measures.

Hepatitis vaccine development is on schedule; vaccine has been administered to volunteers. However, the antigenicity of the vaccine is not as good as hoped for. Attempts will be made to improve this subunit vaccine. In the meantime, epidemiologic studies are continuing and possible populations for vaccine field trials are being identified.

A request for licensure of adenine arabinoside for treatment of herpes encephalitis has been submitted to the FDA, based on studies supported by the Antiviral Substances Program. This drug has also been demonstrated to have some role in reducing signs of chronic hepatitis. There appears to be an additive role when interferon and ara-A are used sequentially. A new promising herpes antiviral, developed by Burroughs Wellcome, has been identified, and will be evaluated as a topical agent against oral mucocutaneous herpes.

Having demonstrated the efficacy of pneumococcal vaccine against bacterial pneumonia, the Program is evaluating its role in otitis media and against pneumococcal disease in patients with sickle cell disease and/or splenectomy. The meningitis program continues in its efforts to obtain a polyvalent vaccine which would be effective in the neonate, and is looking into possible hyperimmunization of prospective mothers as a control measure. Since the current Rocky Mountain Spotted Fever vaccine was taken off the market because of lack of efficacy, the Program is collaborating with the Army in developing and testing a new candidate vaccine.

The Enteric Diseases Program is in its second year, and the three research units established to do definitive studies on vomiting and diarrheal disease in the U.S. are well underway. Utilizing new diagnostic techniques, these epidemiologic studies are expected to yield useful data, including causative agents, pathogenesis, mechanism of immunity, with eventual development of practical means of control.

An important component of the DAB is the Vaccine Evaluation Centers. It is through this mechanism that the specific programs are able to test the various bacterial and viral vaccines under development. These centers are also involved in studies on the pathogenesis and epidemiology of the diseases identified as high priority. There are five such units which complement each other in providing a mix of age groups and types of facilities, i.e., closed or open. Some of these groups are also evaluating antivirals.

Research Highlights

Influenza

The last year has been a very active one in the Influenza Program. The isolation of a new epidemic variant of influenza, the A/USSR/77 (H1N1) virus, stimulated a number of additional studies in response to the public health problems posed by this new virus. Increased efforts in surveillance and vaccine evaluation were accomplished with the collaboration of the Center for Disease Control and the Bureau of Biologics. The Program also undertook field evaluation of antivirals for prophylaxis and therapy of influenza.

Viral Surveillance Activities: The NIAID Influenza Surveillance Network, coordinated through the University of Colorado, was initiated in June, 1976, and has completed its second year. This surveillance effort has produced important data which complement and strengthen the CDC effort in this area. In the initial year of its operation, the NIAID Network of 11 laboratories was the first to detect influenza B activity, and was instrumental in the identification of the newest H3N2 variant, A/Texas/77. During this past year it documented the simultaneous presence of A/Victoria, A/Texas, and A/USSR. Identification of A/USSR has been difficult, probably due to the dependence on eggs for its isolation.

The Influenza Research Center in Houston has continued its in-depth surveillance activities. These studies may provide the best information obtained to date on influenza epidemiology in a large urban setting. The occurrence of three strains concurrently has been well documented, and the isolation of influenza type B during this year is also of interest. Surveillance of animal influenza also continued during the last year through the St. Jude Children's Research Hospital. This group has now catalogued several thousand strains of influenza viruses from avian, swine, and equine species obtained from domestic as well as international sources. Five collaborating laboratories located overseas routinely send isolates to St. Jude's for antigenic analysis and classification.

Vaccine Evaluation Studies: In collaboration with the CDC and the BoB, the Influenza Program has been actively involved in the clinical evaluation of the A/USSR/77 inactivated influenza vaccines. At the time of this writing these studies are still underway, involve 15 research groups, and have enrolled approximately 2200 volunteers. It is through these studies that recommendations on dosage for 1978-79 will be made.

The Influenza Program has also been an active participant in the development and testing of attenuated vaccines. This is a complex project because of the unpredictable variations in the surface proteins of influenza viruses. To deal with this problem, vaccine development has relied on the concept of a master strain which is attenuated. This strain would be used as the donor of the gene or genes required for attenuation to the contemporary wild type strain. Two such master strains have been developed. One is a mutagen-induced temperature-sensitive strain designated as A/Udorn-ts1A2 (H3N2). This strain is markedly attenuated for hamsters and man and carriers two ts mutations. Phenotypically it exhibits a temperature-dependent defect in the synthesis of viral cRNA. Two recombinant clones of A/Udorn were evaluated in the last year, A/Victoria-ts 1A2 (H3N2) and A/Alaska-ts 1A2 (H3N2). The A/Victoria vaccine was shown to be antigenic, immunogenic and extremely well tolerated in volunteers. Recent tests with the A/Alaska-ts 1A2 vaccine have confirmed the high degree of attenuation, although one out of 25 volunteers experienced a non-febrile systemic reaction. The second master strain under study is the A/Ann Arbor/6/60 (H2N2) cold-adapted virus. This virus was derived by successive passage at decreasing temperatures until a strain capable of growth at 25°C was obtained. This virus, like the A/Udorn ts 1A2, is also temperature-sensitive and phenotypically exhibits a temperature-sensitive RNA polymerase activity. Recent studies by Kendal have shown that in over 30 clones of recombinant virus derived from crosses between A/Ann Arbor/6/60 and various H3N2, H2N2 and H1N1 influenza

viruses, four RNA segments coding for RNA polymerase (segments 1 and 3), NP protein, and M protein were reproducibly transferred to the attenuated vaccine strain. Two A/Victoria clones, 19 and 22, have been evaluated in man. Both vaccines were markedly attenuated, immunogenic and provided protection to the vaccinee. These two master strains are approaching the point where comparative field trials for reactogenicity, immunogenicity, and protective efficacy are appropriate. A field trial of attenuated H1N1 vaccine involving 3,000 volunteers is now being planned by the Influenza Research Center for this fall.

An area which requires intensified efforts is the use of influenza vaccines in the pediatric population. This is important both from the standpoint of children at high risk and from the significance of this age group in the spread of epidemic influenza. A study comparing whole-virus and split-virus vaccines is now underway. Also of interest is the development of improved inactivated vaccines for influenza. This research is important because inactivated influenza vaccines do exhibit a reproducible, but extremely low, level of reactogenicity. Such minor discomfort does restrict the use of these vaccines. A subunit vaccine exhibits very low reactogenicity and is a highly purified biologic. Thus, the possibility of introducing antigens not required for a protective immune response is greatly reduced. One vaccine, containing purified hemaglutinin (HA) and neuraminidase (NA), which is called HANA flu, is now under evaluation at the Influenza Research Center.

Antiviral Substances and Influenza: Large-scale field trials were conducted by seven research groups to evaluate the prophylactic and/or therapeutic value of amantadine and/or rimantadine. While the final results of these studies have not yet been analyzed, preliminary indications are that amantadine does, indeed, appear to be effective prophylactically. Although some drug-related side effects have been observed, they were within the tolerated range. Rimantadine does not appear to bear out the early promise of being more effective than amantadine, either prophylactically or therapeutically. The use of aerosolized amantadine requires continued study, but does not look as promising as originally thought. As soon as the data from these studies are more thoroughly analyzed, second phase studies will be planned so that definitive data can be available on the specific role of these agents in the treatment and prevention of influenza.

Respiratory Disease

The magnitude of infection and illness from parainfluenza virus types 1, 2, and 3 and respiratory syncytial virus (RSV) is difficult to document, but estimates can be made based on several epidemiologic studies. There are approximately 3,000,000 infants younger than one year in the United States; about 30,000 (1%) of these infants will require hospitalization for croup. Similarly, 12,000 (0.4%) of the infants will require hospitalization for bronchiolitis and pneumonia associated with RSV infection. The incidence of milder disease resulting from infection with these agents is much greater. Of all respiratory illnesses in infants and young children, 17 percent are estimated to be caused by parainfluenza viruses and RSV; 40 to 50 percent of these cases are severe. Of all cases of pneumonia requiring hospitalization, 40 percent could be prevented if effective vaccines against these agents were available and used.

Vaccine development efforts for respiratory syncytial virus have been conducted primarily by intramural scientists, and a potential attenuated ts mutant is undergoing evaluation. The strain, designated ts-2, has been shown to infect chimpanzees (the most sensitive animal model) and to stimulate protective antibodies against wild type viruses. Tests in a limited number of humans suggest that this candidate vaccine is ready for further evaluation in seropositive children and, if all proceeds well, in seronegative children and infants.

The NIAID has been evaluating a live RSV vaccine developed by Merck Sharpe and Dohme. The vaccine, administered parenterally, is being tested for safety, antigenicity, and efficacy in animal models. The purpose of these studies is to determine if the vaccine should be further evaluated in humans. To date, chimpanzee data suggest that the vaccine is antigenic, but animals with high levels of serum neutralizing antibody are susceptible to wild type virus; the resultant infections are undistinguishable from those in seronegative animals receiving wild type virus.

Considerable progress has been made in the definition of animal models for RSV infection. Simultaneous investigations of the ferret, cotton rat, cebus monkey, and chimpanzee should add considerably to the base information on pathogenesis, immunology, development of vaccine candidates, and evaluation of antiviral substances. Past experience has shown the inactivated vaccines are unsatisfactory immunogens for these viruses, and research efforts should be directed towards the development of live-attenuated vaccines.

A workshop sponsored by the DAB in July, 1977 (J. Infect. Dis. 137:835-846) clearly emphasized the lack of a solid research base for these respiratory disease agents. Epidemiologic and clinical studies to date have not provided answers to questions on correlates of immunity, infection and reinfection, to cite just a few. Accordingly, the MIDP released an announcement for research grant applications for studies on RS and parainfluenza viruses on March 10, 1978.

Hepatitis

The goal of the Hepatitis Program is the control of hepatitis A, B, and non A-non B infections. Acute viral hepatitis is a relatively common illness caused by at least three viral agents. Precise data on morbidity, mortality, and economic burden to the country are difficult to assemble; however, as investigations continue on the etiology, epidemiology, and long-term sequelae of viral hepatitis, it has become apparent that many cases have been misdiagnosed or underreported. In 1975, approximately 55,000 cases of viral hepatitis were reported to the CDC. Allowing for misdiagnosis and underreporting, it can be estimated that 550,000 cases of viral hepatitis occurred in 1975 in the U.S., with a distribution of approximately 50 percent type B, approximately 25 percent type A, and approximately 25 percent type non A-non B. Estimates of death from hepatitis B range from 1,500-3,000 (approximately 1% fatality rate) per year. Costs incurred from hospitalization, professional care, laboratory work, and days lost from employment are estimated to exceed $600 million annually.

Type B hepatitis, previously known as serum hepatitis or post-transfusion hepatitis, has an incubation period of 60-180 days, and may exhibit an acute and chronic phase. Type A hepatitis, known earlier as infectious hepatitis, has a

shorter incubation period of 20-40 days, and apparently does not have a chronic phase. Type non A-non B hepatitis represents agent(s) responsible for approximately 90 percent of the post-transfusion hepatitis remaining in the U.S. Although the virus(es) responsible for this type of hepatitis have not been identified, recent breakthroughs have occurred with the successful transmission of the disease to chimpanzees. Because of its more serious clinical manifestations, type B is a disease of major public health importance in the United States. Efforts to control type B viral hepatitis by active immunization were started after the demonstration in 1968 that the "Australia antigen" (HB_SAg) was associated with the hepatitis B virion. This virus marker allowed studies of virus structure, epidemiology and infectivity in animal models - necessary for the evaluation of the feasibility of developing a hepatitis B vaccine.

During the past year, most chimpanzee experiments with the monovalent adw, ayw, and bivalent adw + ayw vaccines have been completed. The preparations were demonstrated to be safe, antigenic, and highly protective when immunized animals were subsequently challenged with infectious virus. The adw vaccine has been administered to 13 Trappist monks. Each of five monks originally received one dose of vaccine and were carefully followed for six months. The vaccinees had no immediate or delayed reactions and no evidence of infection. Upon completion of the six month follow-up period for the first five volunteers, eight additional monks received tha adw vaccine. Three volunteers received a second inoculation 28 days later. Two of the three developed antibody detectable by radioimmunoassay eight weeks following the booster. Five Trappist monks have been inoculated with one dose of ayw vaccine, so the initial safety test with this preparation is underway.

The antigenicity data accumulated thus far in humans must be considered disappointing, but emphasizes the Institute's step-by-step approach of evaluating all aspects of vaccine feasibility. Areas that will need to be explored in considerable detail include route of vaccine administration, spacing of boosters, and adjuvants. Also pointed up is that data generated from animal models, e.g., the chimpanzee, are not directly translatable to human responses.

Epidemiologic studies in hemodialysis centers, designed to evaluate "at risk" populations for future vaccine efficacy trials have provided interesting data. The incidence of hepatitis B infections in the ten units has been decreasing since the start of the surveillance. This is due to implementation and adherence to proper care of patients and staff within the individual units. Components of proper care are isolation (separate dialysis machines) of carrier patients, assignment of staff with anti-HB_S to carrier patients, handwashing, and control of environmental factors. The attack rates may be at stable levels now, or they may decline further, but additional surveillance is needed to ascertain this.

While efforts at vaccine development for hepatitis B virus are well underway, it is recognized that a major gap in hepatitis research exists since none of the agents of viral hepatitis have been propagated in vitro. The NIAID is supporting efforts to develop substrates (human and chimpanzee hepatocyte cultures) for intensification of culture efforts.

Animal models have contributed tremendously to efforts in viral hepatitis research; and in collaborative efforts with the BoB/FDA, breeding colonies of chimpanzees and marmosets are supported so that Institute scientists have animals available for their studies. The role of antivirals is also being assessed and is described in the Antiviral Substances Program section.

Antiviral Substances

This Program played a major part in recent studies which demonstrated the effective role of antivirals in the clinical setting. The need for antivirals in the control of infectious diseases remains critical. Although vaccine prophylaxis remains the first line of defense against many viral diseases, there are many areas where vaccines are not available or feasible. Chemotherapeutic/prophylactic agents with low toxicity and high rate of effectiveness are needed. At present there are only three antivirals licensed for use in the United States: idoxuridine and adenine arabinoside for herpes keratitis, and amantadine for influenza A prophylaxis. It is anticipated that adenine arabinoside will be licensed for use against herpes encephalitis within the next several months.

During the past year two important workshops were held. The Workshop on Clinical Trials with Exogenous Interferon, jointly sponsored with NCI, was held on March 21-23, 1978. This was the first meeting at which all studies throughout the world were reviewed. The effectiveness of exogenous interferon against herpes zoster was demonstrated in a double-blind study. Such interferon has a definite effect on modification of the carrier state of type B hepatitis, but its exact role is yet to be defined. Other studies are addressing dosage regimens and combined therapy with other drugs prior to initiating a proper double-blind, placebo-controlled study. Work presented by Strander and others indicated that interferon may be an effective treatment for a variety of metastatic diseases, including osteogenic sarcoma, Hodgkin's disease and multiple myeloma; these findings should be confirmed by double-blind controlled studies.

To date most antivirals have been developed through huge screening programs which are costly and lead to antivirals purely through chance. Thus, on June 29-30, 1978, a meeting was held to discuss better ways to develop antivirals. The questions posed were: is the molecular biology of viral replication at the point where specific viral enzymes and proteins can be identified, and can the chemists produce specific inhibitors? The answers to both questions were affirmative. It is up to the ingenuity of the program and the scientific field as to how this is to be accomplished through coordination of efforts of virologists, chemist and pharmacologists.

Interferon: The use of interferon to treat herpes and hepatitis infections is being studied at Stanford University. Interferon at 5.1×10^5 units per kilogram per day for up to five days has been shown to significantly diminish severity of post-herpetic neuraligia, decrease acute pain and limit cutaneous dissemination, progression within the primary dermatome and visceral complications (N. Engl. J. Med. 298:981-987, 1978). The role of interferon in varicella disease is currently being investigated.

It has been estimated that there are 175 million people in the world who are chronic carriers of hepatitis B, approximately 10 percent of patients hospitalized

with acute hepatitis B in the U.S. become chronic carriers. They, in turn, are potential reservoirs of infection as well as candidates for liver cancer. Interferon appears to have a role in the eradication of the carrier state. The Stanford group is conducting studies designed to determine the optimal regimen for a treatment in a double-blind, placebo controlled study. Seven patients have received a potentially eradicative treatment schedule with human leucocyte interferon. Of these, three patients have shown permanent reduction of Dane particles and HB_eAg to below the level of detection and partial or complete reduction of HB_cAg in the liver. All patients have shown a reduction in the Dane particles following interferon treatment, but permanent reduction or complete eradication of the disease is not always possible, particularly in postmenopausal women and in men. This group has also investigated the use of adenine arabinoside (ara-A). Of four patients receiving a regimen of ara-A, two patients have shown a permanent reduction of Dane particles and HB_eAg to below the level of detection, partial reduction of HB_sAg, and disappearance of HB_cAg from the liver biopsy. Currently, sequential treatment with interferon and ara-A is being investigated. It is expected that a double-blind trial will be initiated shortly to test the most promising therapeutic regimen.

Surgery to alleviate trigeminal neuraligia has been shown to precipitate serious herpes labialis. Investigators at the University of Pittsburg are evaluating the use of interferon in a prophylactic regimen. Initial analysis of the data indicates that interferon will reduce the incidence and severity of these lesions.

Other Antivirals: The effectiveness of ara-A in reducing the mortality of herpes encephalitis from 70 to 28 percent is well accepted, and the drug is being considered for licensure by the FDA. Studies are continuing on the effectiveness of ara-A against herpes zoster, neonatal herpes and severe herpes hominis infections. These protocols are being evaluated throughout the United States with the University of Alabama serving as the central coordinating unit. Adenine arabinoside monophosphate (ara-AMP), a more soluble analog of ara-A, is being studied to determine if active levels of this compound can be attained in humans. If this can be accomplished, a study will be initiated comparing ara-AMP to ara-A against herpes encephalitis.

A critical part of the Program's effort to develop clinically effective antivirals has been the establishment of a series of animal-viral disease models which closely mimic viral diseases of man. Five contracts provide six species of animals (mice, rabbits, guinea pigs, cats, pigs and monkeys) and a variety of DNA and RNA viral infections to evaluate rapidly the spectrum of a promising compound. The most promising compound evaluated this past year was 9-(2-hydroxyethoxymethyl)guanine, BW-248U, an antiviral developed by Burroughs Wellcome. This compound is currently undergoing preliminary studies prior to initiation of clinical testing against oral mucocutaneous herpes. BW-248U is a nucleoside derivative which structurally resembles 2-deoxyguanosine, is converted to a monophosphate by a virus-specified thymidinekinase, and subsequently to di and triphosphates. In the uninfected cell these phosphorylations do not occur. BW-248U inhibits DNA polymerase and acts as chain terminator of the viral DNA by incorporation of acycloguanosine. Once the antiviral gets into a cell and is phosphorylated, it appears to stay trapped within the cell. If this drug

continues to show promise, it will be considered for systemic use against herpes encephalitis and eventually herpes genitalis.

Bacterial Vaccines

Pneumococcal pneumonia: Through NIAID contracts, polyvalent vaccines of pneumococcal capsular polysaccharides have been developed, and clinical evaluation of these vaccines has been conducted over the past several years. Two efficacy trials of the vaccine in preventing pneumococcal pneumonia were sponsored in the U.S., and the program collaborated in trials in South African gold miners.

The South African studies showed that penumococcal vaccine was 79 percent effective in preventing radiologically confirmed putative pneumococcal pneumonia and pneumococcal bacteremia due to types in the vaccine. The vaccines were 83 percent effective in preventing type specific bacteremia. Using the same diagnostic criteria, the disease incidence was not high enough in the U.S. studies to show a high level of effectiveness of the vaccine. Inclusion of seroconversion in the criteria for diagnosis of type specific disease, however, yielded data demonstrating that the vaccine was approximately 90 percent effective.

Based on these data and data supplied by Merck Sharp and Dohme, the BoB granted a license to Merck Sharpe and Dohme for the manufacture of a 14-valent pneumococcal polysaccharide vaccine. The current recommendations for use include those individuals over age two years who are considered at high risk to pneumococcal infections.

During the past year, the Bacterial Vaccines Program has continued to collaborate (without direct funding) in studies of patients with various conditions which predispose them to pneumococcal disease but in which data are insufficient to warrant specific recommendations for use of vaccine. The patient conditions under study include sickle cell disease patients, splenectomized patients, bone marrow transplant recipients, Hodgkin's disease patients, renal allograph recipients, and chronic dialysis patients. Lymphoma, myeloma, lupus, lymphoblastic leukemia, alcoholic cirrhosis, and chronic obstructive pulmonary disease patients are also being studied. It is hoped that these studies will lead to expansion of the recommended uses of the vaccine.

Meningitis: It is estimated that approximately 20,000 cases of bacterial meningitis occur in the U.S. each year, most of which occurs in young children and infants. The meningitis vaccine program is concerned primarily with bacteria causing meningitis in children.

A candidate H. influenzae type b vaccine, consisting of purified polyribose phosphate (PRP) capsular material, has been developed. Results of clinical studies show that children over 18 months of age respond well to the pure PRP vaccine; infants under this age respond poorly or not at all. The H. influenzae PRP vaccine was used in the field trial of meningococcal A polysaccharide vaccine in Finland (described below), and data from that study show that the vaccine is highly effective in preventing H. influenzae disease in children over 18 months but ineffective in younger children. The Finnish data also indicate that booster doses in the older children may improve the long-term persistence of protective antibody levels.

A current objective is the development of alternate antigenic preparations which may be more immunogenic in infants. An investigator at the University of Rochester has developed a new candidate vaccine for H. influenzae disease in infants. This preparation is a complex of PRP, several polypeptides, and lipopolysaccharide, and shows promise in animal studies. Preliminary safety and immunogenicity studies of this vaccine (designated PRPc) have been done in adults, and studies in older children will be starting in the near future.

With bacterial polysaccharide vaccines, larger molecular weight is consistently associated with improved immunogenicity. Studies have also been directed toward the development of higher molecular weight PRP preparations. It is anticipated that one such preparation will be used in phase one clinical studies in the near future.

In addition to efforts to improve the infant's response to bacterial meningitis (particularly H. influenzae) vaccines, either through modification of the antigen or the infant's immune response, protection of the neonate and young infant by hyperimmunization of the mother is being pursued. A study is currently in progress in which H. influenzae PRP is being given to women who plan pregnancies and to a control group of women who do not plan to become pregnant. Serum samples will be taken prior to delivery, from cord bloods, and from infants over a period of time to determine if the level of infant antibody to H. influenzae can be increased during the crucial first few months of life.

Group A and C meningococcal polysaccharide vaccines have been licensed for use in adults. The group A vaccine has also been shown to be effective in children (see below) and is recommended for use in epidemic situations. Group C meningococcal polysaccharide vaccine has not been shown effective in children and, in fact, antibody response in infants is not very good. A new polysaccharide extracted from a variant group C strain has been prepared at Rockefeller University. The chemical structure varies somewhat from the current vaccine, but is closely related antigenically. In clinical studies in adults, this vaccine was shown to be superior to the current C vaccine in immunogenicity. Studies are beginning in children and infants.

In 1974 a contract was awarded to the Central Public Health Laboratory, Helsinki, Finland, to conduct an efficacy trial of the group A meningococcal polysaccharide vaccine in children ages three months to six years. H. influenzae PRP was selected as the control vaccine. The results from the trial show that group A meningococcal vaccine was highly effective in preventing group A disease throughout the entire age group. As mentioned previously, the H. influenzae vaccine also showed effectiveness in the age group over 18 months but was ineffective in the younger children.

Important data on the persistence of antibody have also been obtained in the Finnish study. All children 18 months and younger received boosters three months following the primary injections. In the children at the upper age range of this group (i.e., those 17-18 months of age), higher antibody titers did not occur following booster; however, it appears that persistence of protective levels is much greater after two years than in children 19-20 months old who receive only one injection. The Finnish contract was renewed for an additional

three years to further study persistence of antibody levels and to permit additional booster studies.

Group B meningococcal polysaccharide is non-antigenic in humans. A protein antigen vaccine candidate, from the outer membrane layer of the organism, has been developed by the BoB. Animal safety and immunogenicity tests have been completed, and a limited number of humans have received the candidate vaccine. The NIAID will initiate phase one safety and immunogenicity studies in adults in the near future. A second cell envelope protein antigen prepared at the Naval Medical Research Institute is also under consideration for human studies.

A surveillance study to determine the incidence and responsible causal organisms of bacterial meningitis in Los Angeles County was conducted during calendar years 1975 and 1976. This project was undertaken to determine if it would be feasible to conduct an efficacy trial of H. influenzae vaccine in the U.S. The Southeast health region in LA County was identified as suitable for a large scale field trial based on the H. influenzae incidence data. The baseline data provided could form the foundation for a future efficacy trial.

Otitis Media: Acute otitis media is a very important childhood disease. Almost every child has otitis media at least once, and 20 to 30 percent have six or more occurrences. Pneumococci are estimated to be responsible for 40 to 50 percent of all cases. A program to study the value of pneumococcal polysaccharide vaccines in preventing otitis media was initiated in 1975 which includes two double-blind efficacy trials of an octavalent vaccine for the prevention of disease in children who have had previous middle ear infections. The vaccine code has not been broken so no efficacy data are currently available. A third project is designed to further establish the optimum dose and booster regimen for maximum antibody response of infants and young children. Using the octavalent preparation, variable response to the individual polysaccharides has been seen. Some polysaccharides (type 3, for example) simulate response even at six months. Other types, including 7 and 18, give measurable increase when given at 6 and 12 months, while other types are poorly antigenic with any dose regimen. These studies will be continued using the recently licensed Merck 14-valent preparation. There are currently little data on this vaccine in U.S. infants.

To better understand the pathogenesis and immunology of pneumococcal otitis media and the efficacy of pneumococcal polysaccharide vaccine in preventing experimentally induced middle ear disease, an animal model system has been developed. Experimental infection can be induced by direct inoculation of bacteria into the middle ear cavity of chinchillas. A further refinement has allowed the investigator to induce pneumococcal otitis media following colonization of the nasopharynx. This latter challenge system will allow for studies of disease more closely resembling human otitis media. Using this model, vaccination with pneumococcal polysaccharides was shown to reduce bacteremia and mortality.

Rocky Mountain Spotted Fever Vaccine: The presently licensed vaccine for RMSF is not considered effective and is no longer commercially available. A new inactivated, whole-cell vaccine has been developed at the U.S. Army Research Institute for Infectious Diseases. Because of our mutual interest, the NIAID began a collaborative effort with USAMRIID which has continued into FY78. The NIAID

is supporting phase one clinical trials of the vaccine in adults at USAMRIID facilities at Fort Detrick. The NIAID wishes to follow the adult studies with safety and antigenicity studies of this vaccine in children age 2 to 16 years.

Enteric Diseases

The mortality and morbidity due to diarrheas has a staggering impact on the economy of the world. In a well developed area such as North America, diarrhea is the eighth leading cause of death in children one to four years of age with a rate of 1.4/100,000. It is also among the ten most common causes of visits to the doctor's office or of hospital admissions. Worldwide it is estimated there are 500 million cases of gastroenteritis per year, with an estimated 5-18 million deaths in children under five years of age in Asia, Africa and Latin America.

Enteric Disease Study Centers: With the reorganization of the Institute, the Enteric Diseases Program was initiated. Its long-term goal is to prevent or minimize these diseases by epidemiologic or public health techniques and/or immunogens. Three Study Centers were established to examine the epidemiology of the various causes of diarrhea, and to clarify their pathogenesis.

The three Enteric Disease Study Centers are: D. C. Children's Research Hospital, Washington, D.C., studying predominantly an inner city population; the University of Michigan, Ann Arbor, studying a small town and rural population; and the University of Texas, Houston, working in a mixed ethnic urban population. Their efforts are divided into three phases: (1) defining the scope of the problems caused by all known etiologic agents by epidemiologic techniques; (2) identifying the pathogenesis of each disease, especially the newly discovered viral agents; and (3) identifying and testing practical means of control.

Agents of Particular Interest: The recent development of techniques for identifying rotaviruses and a 27 nm particle associated with "winter vomiting" disease opens a whole new avenue of work. These agents, and perhaps other similar agents, appear to be significant causes of diarrhea, especially in children under five years of age. Progress in control of these agents is handicapped by the lack of effective techniques for *in vitro* cultivation.

The major portion of enterotoxin mediated diarrheas in the U.S. is due to enterotoxigenic *E. coli* (ETEC). These organisms are commonly associated with travelers diarrhea occurring in any and all Americans traveling to other parts of the world, especially into other cultures. Their occurrence in U.S. communities is less predictable. Studies at the University of Virginia indicated a small role for ETEC in episodes of acute diarrhea in the local population, but these organisms played a significant role in diarrhea in residents of Brazil, in a Navajo reservation, and in North American travelers to Central America. In these latter groups, heat stable enterotoxin (ST) producing *E. coli* appeared to be more important than did the heat labile (LT) strains. This is in contrast to some earlier information which indicated LT strains were important causes of diarrhea in U.S. travelers to Mexico City. The incidence of disease appears to be related to environmental sanitation.

Synthesis and partial purification of the LT have been accomplished. Various strains do not appear to be identical immunologically although they are antigenically similar. The molecular mechanism of LT action is very similar to that of cholera toxin. Immunization of rats with cholera toxoid produces protection against enteric challenge with LT. The ST toxin, in its present form, is a poor immunogen.

Evidence of specific proteins associated with bacterial colonization of the intestine has been found. These "colonization factors" and their relation to disease are being investigated. Clones of certain E. coli fed to volunteers in large numbers caused a brief, acute, watery diarrhea even though they were noninvasive and enterotoxin negative. These organisms may have an as yet unexplained or unidentified mechanism for causing diarrhea. One strain (RDEC-1) which is noninvasive and produces neither ST nor LT, has been found to produce small amounts of an enterotoxin similar to or identical with S. dysenteria enterotoxin. The causal relationship to diarrhea is unknown at this time.

Cholera: The seventh world pandemic in cholera continues with 34 countries reporting cases in 1977 as opposed to 25 in 1976. Of particular concern was the spread to the Gilbert Islands in the Pacific Ocean. Cases reported from developed countries were all imported. The Institute program in cholera is part of the U.S.-Japan Cooperative Medical Sciences Program. Cholera studies are important per se, but also serve as an excellent model for studies on enterotoxin mediated diarrheas including studies of the local immune response.

Cholera challenge studies are continuing in volunteers. The solid protection reported last year in volunteers who had induced cholera was shown to last for eight to ten weeks, and was effective against either homologous or heterologous serotypes. The protection appeared to include a highly effective antibacterial mechanism since vibrios could not be recovered from feces or duodenal fluid after their first challenge. The next step will be to rechallenge at an interval of 12-24 months. These studies suggest an important and effective role of enteric immune mechanisms stimulated by local antigen exposure, and provide impetus to attempts to achieve or exceed such protection by immunizing procedures.

The Wellcome Foundation Limited, Beckenham, Kent, England, is cooperating with the program by providing a combined whole cell vaccine and purified toxoid mixture for field testing. The safety of these products was conducted at the University of Texas facility, and the field testing will be done at the Cholera Research Laboratory in Dacca, Bangladesh. This area has endemic cholera and is well suited and established as a field test facility.

Studies on antibacterial and antitoxin immunity in animals have focused on the local immune system of the intestine. In dogs, a local antitoxic response is induced by parenteral priming with a single dose of purified toxoid followed by multiple oral doses of toxoid. The mechanism of protection, however, is uncertain since the protection far outlasts the presence of antitoxin-producing plasma cells in the intestinal mucosa. Oral doses of crude toxin protect against live vibrio challenge also, but oral doses of toxoid do not. This is consistent with earlier studies in rats, and provides a valuable model in which to study mucosal immunity to cholera provided by oral immunization. The mechanism of this immunity appears to be mediated by both humoral and cellular mechanisms. The

immunization route and antigen form appear to be important determinants of the type of response obtained. In some instances there is a suppression of local IgA immune response. A better understanding of the mucosal immune response in animals would have important implications for human protection.

Basic studies on the structure and function of cholera toxin have provided information on the mechanism of action of cholera toxin. The major effect is adenyl cyclase activation. The process appears reversible; however, studies in rabbits continue to demonstrate that nicotinic acid reverses the secretory and adenyl cyclase-activating effects of cholera toxin. The exact mechansm of action and possible therapeutic value are as yet unknown.

Colonizing factors, motility and enzymes continue to be areas of interest in relation to virulence. Genetic manipulation is being tried to produce an avirulent oral immunogen.

BACTERIOLOGY AND VIROLOGY BRANCH

The Bacteriology and Virology Branch plans and conducts research grant, program project grant, career award, training grant, fellowship and contract programs in Bacteriology, Mycology, and Virology. Dr. William P. Allen administers the Virology Program of the Branch. He is responsible for a considerable research and training program covering the broad spectrum of fundamental virologic investigations. These deal with clinically important viruses as well as those serving as models of viral infections. In addition, he coordinates the activities of the Viral Diseases Panel of the U.S.-Japan Cooperative Medical Science Program. Dr. Darrel D. Gwinn has replaced Dr. Paul D. Lambert (retired); he coordinates the activities of both the Leprosy and Tuberculosis Panels of the U.S.-Japan Cooperative Medical Science Program. He will also administer the Institute's new program initiative in Mycology.

Approximate Level of Support

Bacteriology and Mycology

Activity	Number	Amount
Research Grants	135	$10,466,204
Career Awards	15	528,892
Training Grants	20	1,574,783
Fellowships	11	155,200
Research Contracts	7	470,562
Total	188	$13,195,641

Virology

Research Grants	164	$13,704,221
Career Awards	20	671,047
Training Grants	15	1,010,433
Fellowships	23	282,400
Total	222	$15,668,101

Branch

Total	410	$28,863,742

Bacteriology, Mycology and Virology

Program Summary

Advances in fundamental knowledge developed in the basic sciences are the cornerstones upon which medical application to alleviate specific health problems ultimately rests. This Program supports projects in both fundamental and applied biomedical research, with the ultimate goal being the translation of this knowledge into firm methods for the diagnosis, prevention and therapy of a wide array of bacterial, fungal and viral diseases.

Interest in certain targeted research areas is continuing, and it is the objective of the Branch to maintain and expand them where possible. The ongoing special programs in Bacteriology are those in Sexually Transmitted Diseases, Hospital Associated Infections, and Streptococcal Diseases and Sequelae. A new program initiative in Mycology was approved by the National Advisory Allergy and Infectious Diseases Council. This resulted from recommendations made by experts in this area who participated in a workshop on Mycology under the auspices of the BV Branch.

The impact that viral infections exert on human health is substantial, and control of these infections is the ultimate goal of the Virology research supported by the NIAID. The following programs are highlighted and receive emphasis: Clinical Virology, Persistent Infections and Chronic Viral Diseases, and Biology of Viruses. The Virology program also supports targeted research on rabies, dengue, and other arthropod-transmitted diseases under the U.S.-Japan Cooperative Medical Science Program.

Bacteriology

Sexually Transmitted Diseases (STD)

Sexually Transmitted Diseases (STD) are still considered one of the major public health problems, with gonorrhea classed as a national epidemic. The most recent statistics indicate, however, that there is now a slight decline in the rate of increase of reported cases of gonorrhea and of syphilis, for unknown reasons. Cases of herpes type 2 virus seem to be increasing; this is also true for nongonococcal urethritis cases, although definitive data are lacking. The Institute's program on STD was funded during this past period at approximately $5.5 million; this included contracts, training programs, and grants. There is widespread interest in this research program, although the number of people in specialized training or seeking such training for research careers in this area is not large. M.D. candidates, especially, seem to be reluctant to undertake postdoctoral training in infectious diseases.

Research Highlights

NO1 AI 52535 T. J. Buchanan (University of Washington): One of the long-standing needs in our efforts to understand and to control the gonorrheal epidemic has been the lack of a satisfactory serotyping system. Dr. Buchanan has studied the isolation and characterization of several antigens from the gonococcus to develop a practical system for serodiagnosis. He has

investigated antibody elicited by the gonococcal pili, the principal outer membrane protein (POMP), and the lipopolysaccharide. Results of these studies show that antibody to pili was not bactericidal but was opsonic, whereas antibody to POMP was bactericidal but had no opsonic activity. In contrast to the more than 30 serotypes of gonococcal pili, there seems to be a very limited number of POMP gonococcal serotypes. Of gonococci isolated from cases of disseminated disease, a majority were of a single POMP serotype. The presence or the function of capsular antigen of the gonococcus has not yet been definitely established. The group under Dr. Buchanan has chosen to develop the enzyme-linked immunosorbent assay (ELISA) as the primary technique with which to test the suitability of the various antigens in a serotyping system. The antigenic heterogeneity of pili has been confirmed by the pili-ELISA system. This system can be of value in any future trials of POMP and pili as candidate vaccine material in humans. An Investigative New Drug (IND) application for this material is now pending for projected Phase I testing.

AI 13488-03 Z. A. McGee (Vanderbilt University): The pathogenesis of gonorrhea has been difficult to study for lack of a suitable non-human model. Dr. McGee is studying the mechanisms by which gonococci cause disease in the human genital tract by means of an organ culture model system using human fallopian tube sections. The rate of damage to the mucosa can be quantitated and the interaction with the gonococci studied microscopically. Data obtained show that attachment of gonococci may enhance mucosal damage by a toxic factor. This toxic factor is present during the first 24 hours of infection, and remains at a high level thereafter. The toxic factor appears to be an endotoxin, and is responsible for at least part of the damage to the recipient cells. There is also a strong indication that the toxin(s) is specific for human genital mucosa and not for that of other species. Of interest also is the finding that type 4 gonococci, which lack pili, nevertheless do attach to fallopian tube mucosal cells, albeit more slowly than the piliated type 1 organisms.

NO1 AI 42536 A. H. Fieldsteel (Stanford Research Institute): One of the major problems in research in syphilis is ability to grow the causal organism, Treponema pallidum, in vitro. Dr. Fieldsteel has made some useful approaches that may be of great value in future growth studies. He has developed a medium and tissue culture system in which he demonstrated T. pallidum survival and motility retention for up to 21 days, with concommitant retention of virulence. Growth in rabbit testis tissue culture was critically dependent on ambient gas atmosphere, with low concentrations of oxygen being beneficial. Dr. Fieldsteel also has found, with an I^{125} labeling technique, that there is no detectable homology between virulent T. pallidum (Nichols) and each of five strains of cultivable treponemes. These treponemes are therefore genetically distinct and are not variants or mutants.

AI 13069-03 A. L. Barron (University of Arkansas): Chlamydia trachomatis is a causal agent of nongonococcal urethritis (NGU), the second most prevalent of the STDs. In the male it causes NGU, while in the female the infection is primarily a cervicitis. Newborns of infected mothers frequently develop inclusion conjunctivitis and the recently described infant pneumonia syndrome. Dr. Barron is studying the immune responses of chlamydial infections in a guinea pig model infected with a chlamydial inclusion agent

(gp-ic). The effect of the immunosuppressant drug Cyclophosphamide (Cy) on both humoral and cellular immunity was investigated. When both B and T cells were depressed by Cy, the infection persisted for 35-45 days; the T cell function returned by day 15, but antibody to gp-ic was not detected until day 21 when it remained at a high level. Recovery from infection was coincident with antibody development. Treatment with higher doses of Cy resulted in selective suppression of B cells only, and animals remained infected until death at day 30. No antibody response was detected in these animals. In Cy-treated animals, an ascending genital infection, including endometritis and cystic salpingitis was seen. The data indicate that in the presence of a functional cell-mediated system animals fail to recover in the face of a suppressed humoral antibody response. In other infection experiments, results showed that involvement of guinea pig vaginal tissue was superficial, but an intense inflammatory response of the exocervix was observed; no evidence of endometrial infection was seen.

Conferences

A conference on "Immunobiology of Neisseria gonorrhoeae" was held in January 1978 to discuss the latest advances growing out of gonococcal research. There are still many questions regarding the microorganisms and the disease that remain to be answered, as was amply demonstrated at the conference. Research progress in this area, however, has been moving forward at an accelerated pace. A great deal of interest in this problem is indicated by the large audiences in attendance at this and similar meetings devoted to research on gonorrhea. The conferees also discussed the possible development of a gonococcal vaccine; this may well become a reality, although all the definitive information is not yet at hand. The conference proceedings will be published as a book in 1978, under the auspices of the American Society for Microbiology.

Streptococcal Diseases and Sequelae (STP)

Group B streptococcal infections (GBS) are one of the leading causes today of mortality or serious sequelae in neonates. It is well known that many expectant mothers at term have low GBS antibody levels, and that they may also be carriers of GBS in their genital tract. Infants born to these mothers consequently are at high risk of developing a GBS infection either at birth or shortly thereafter. This research program is currently funded at approximately $1.7 million, and includes grants, contracts, and training programs.

Research Highlights

AI 13249-02 C. J. Baker (Baylor University): Dr. Baker and her collaborator, Dr. Dennis Kasper (Harvard), have isolated and purified polysaccharides from type III GBS. They are now studying the development of antibodies in adults selected for existing low concentrations of natural antibody to the different antigen preparations. These were purified by different methods for potential use as candidate vaccine materials. None of these preparations showed any pyrogenic or toxic activity. A single 50 Mcg subcutaneous injection in human volunteers elicited significant increases in antibody concentration; these responses were maximal in two weeks and remained elevated at 21 weeks

post-immunization. The vaccine-induced antibody was of the IgG class. These workers, and others, are looking toward methods of preventing GBS infections. One means of prevention would be by immunizing the expectant mother, with the goal of developing a high antibody level in the mother and subsequently in the fetus; another potential method would be by passive immunization of the neonate with high-titered, purified anti-GBS human IgG.

AI 13926-01 P. Ferrieri (University of Minnesota): Dr. Ferrieri has developed a rat model to study the pathogenesis and epidemiology of GBS infections. Results show that mortality of rat pups to type III GBS is dose dependent as well as age dependent; older animals (15 days) are more resistant than younger ones. Epidemiology studies of adult human females have shown a high rate of rectal and genital carriage of GBS; this was also investigated in the rat model. Oral feeding of $1x10^7$ microorganisms to three-week-old rats resulted in 71% positive rectal cultures; intra-litter spread was also common. Vaginal colonization of adult virgin rats resulted in positive colonization with types II and III GBS in 23% of the animals. In other studies, parturient women with positive genital tract cultures as well as cord blood from their infants have been examined for streptococcal anti-nuclease activity. Neutralizing activity for the GBS nuclease was detected in 25% of the maternal and cord sera, as compared to control groups which showed very low levels of activity. These results can aid in documenting serologically the host responses to GBS antigens in future studies of vaccine response.

PO1 AI 13464-02 J. H. Schwab (University of North Carolina): Drs. Schwab, Cromartie and their team have been testing a unique hypothesis, that bacterial cell wall fragments are toxic and play a role in rheumatoid arthritis and rheumatic fever. They are studying this problem using a rat model injected with isolated cell wall fragments of Group A streptococci. Joint involvement in these animals is pronounced; the rheumatoid sequelae in the rat are evaluated by clinical, radiological and histological methods. The incidence and severity of joint disease is dose related; typically, an acute inflammatory process after injection is followed by soft tissue swelling and joint involvement. No correlation was seen between cell-mediated immunity (CMI) as measured by delayed hypersensitivity to Group A streptococcal cell walls and joint disease. Additionally, no correlation was found between antibody levels against peptidoglycan or polysaccharide antigens of the streptococcal cells and the joint disease. The arthritis induced by the streptococcal cell wall fragments in the rat model is similar in many respects to human rheumatoid arthritis.

PO1 AI 08724-22 L. W. Wannamaker (University of Minnesota): In this program project Dr. Wannamaker is studying the biology of both Group A and B streptococci. A bacteriocin of Group A streptococci was isolated and characterized; it was of low molecular weight (8,000 daltons) and nonantigenic. It also inhibited the syntheses of DNA, RNA and protein, and prevented the uptake and incorporation of glucose into sensitive cells. Loss of ability to produce the bacteriocin was associated with loss of immunity to its killing action. A bacteriocin from Group B streptococci was also identified. This existed in two distinct and interconvertible forms; it was heat stabile but inactivated by proteolytic enzymes. In addition, three separate nucleases

have also been identified from Group B streptococci; they have a molecular weight ranging from 18,000 to 35,000 daltons and are immunologically distinct from those of Group A streptococci. The cytotoxic effect of streptolysin O on myocardial cells was also investigated. The addition of very small amounts of streptolysin O to beating mouse myocardial cells in Sykes-Moore chambers produced toxic changes (cessation of beating and cell lysis). These cytotoxic changes were blocked by lipids from rabbit skin and by cholesterol. These studies suggest, therefore, that cholesterol may have a protective effect against streptolysin O toxic action.

Hospital Associated Infections (HAI)

The major problem in Hospital Associated Infections (HAI) has shifted in the past 15 years to that of the endogenous (resident) gram negative microbial flora. These have now become prominent because they have achieved antibiotic resistance; the number of immunosuppressed patients, susceptible even to their own gut bacterial flora, has also increased dramatically. A high incidence of bacteremia, with a high mortality rate, is the result. This special program in HAI is currently providing $3 million of support for grants and training programs.

Research Highlights

AI 10108-08 A. I. Braude (University of California, S.D.): This investigator has developed a therapeutic antiserum of great potential for treating patients with life threatening gram negative bacterial infections. In earlier reports he described the J5 antiserum produced in humans vaccinated with the core glycolipid from an E. coli strain. In a double blind study of patients in profound bacteremic shock, he found that recovery from shock in the J5 serum treated group was nearly three times that of the control group. Tests in rabbits given the J5 antiserum, to study optimum dosage against fatal Pseudomonas sepsis, indicated that protection was not dose dependent, and was undiminished after two weeks. The J5 antiserum administered prophylactically to animals prior to inoculation with Pseudomonas provided as strong protection (75%) as antiserum given for therapy against an established bacteremia. The feasibility was therefore established of using J5 human antiserum prophylactically in patients with a high risk of developing gram negative bacteremia.

AI 08504-09 P. F. Bartell (New Jersey College of Medicine): One of the major problems in cystic fibrosis patients is the severe infections with Pseudomonas aeruginosa that often accompany the disease. The pathogenic role in this disease of the slime layer of the organism is still undetermined. Dr. Bartell has isolated and purified a glycolipoprotein (GLP) from this slime, and has shown that it acts as a toxin and as a protective antigen. In a rabbit model, immunization with GLP resulted in hemagglutinating and bacterial agglutinating activity. These paralleled mouse passive protection activity against lethal challenge with the Ps. organism; the protection was dependent on activity levels in the plasma. The activity was found in the IgM serum antibody fraction. This suggests that the GLP may offer a potential means of passive protection against the toxic and lethal effects of Ps. aeruginosa infections in cystic fibrosis patients.

AI 13120-02 C. D. Cox (University of Iowa): A different type of study in Ps. aeruginosa infection is that of Dr. Cox, who is exploring the role of essential nutrients and the competition between mammalian tissue and the organism for nutrient iron and for dissolved oxygen. The principal investigator has found a new pigment, pyochelin, in Ps. aeruginosa cultures; this is classed as a siderophore because of its dramatic growth stimulation of the organism under iron-limiting conditions. All clinical isolates of Pseudomonas examined so far contain it. Preliminary data on the role of iron in a mouse model system suggest that phagocytic cells and nutritional immunity of the host may be the main defense mechanisms during the initial stages of infection. It is also considered possible that passage of Ps. aeruginosa strains through patients in a burn ward may affect selection and adaptation of the organism toward iron metabolism.

AI 11950-04 J. P. Burke (University of Utah): A major public health problem is that of hospital acquired urinary tract infections secondary to the use of indwelling bladder catheters. Dr. Burke has been studying methods for prevention and control of such nosocomial urinary tract infections. Previous data showed that a newly designed closed drainage system offered no better overall protection than the currently used closed systems. Dr. Burke has determined that the major pathway for bacterial migration into the upper urinary tract is through the mucous sheath surrounding the intraurethral portion of the catheter. The use of oral trimethoprim sulfamethoxazole (TMP-SMZ) therapy for eight days in catheter patients significantly reduced the level of infection and prevented bacteriuria during indwelling catheter drainage.

AI 12936-01 J. W. Alexander (University of Cincinnati): Nosocomial infections in surgical and burn patients are being studied for immunologic variables of host resistance to infection. Infected burn patients had significantly worse neutrophil bactericidal activity; this was consistently observed in association with infection. In the bacteremic episodes, immunologic measurements in the burn patients showed significant predisposing abnormalities, primarily of neutrophil function and opsonization activity. Kidney transplant patients are in a similar category, with transient defects in the primary defense mechanism. In a guinea pig model, malnourished individuals could not maintain adequate levels of C3 complement factor. Patients are therefore at much greater risk when malnourished because of the failure of synthesis of the C3 complement factor. Nutritional support may therefore be a vital factor in host response to infection in these patients.

AI 14789-01 W. R. McCabe (Boston City Hospital): Previous studies have demonstrated that both active and passive immunization with the Re mutant of Salmonella minnesota protected mice against lethal infections with K. pneumoniae and P. morgani. Antibody to the Re mutant protects against both initial bloodstream invasion and lethality of the bacteremia. Studies in volunteers were recently initiated after completion of extensive animal testing and Food and Drug Administration approval of an Investigational New Drug (IND) was obtained. Intradermal injection of 10 Mcg of Re lipopolysaccharide showed a significant increase in antibody titers to the Re mutant, with no adverse systemic reactions observed. Subcutaneous immunization with the Re mutant was also initiated. No systemic reactions were seen and local arm reaction was equivalent to that seen after typhoid vaccination. These volunteer studies are continuing.

Leprosy

Leprosy, a major health problem of the developing countries, occurs with a very low incidence in the U.S., about 0.5 cases per million. Because of its worldwide importance, and its usefulness as a model of an immunological disease, however, leprosy research continues to be supported by NIAID under the auspices of the U.S.-Japan Cooperative Medical Science Program. Research being supported includes *in vivo* and *in vitro* cultivation of *M. leprae*, pharmacology of antileprosy drugs, chemotherapy trials and studies of the basic immunology of leprosy.

Research Highlights

AI 14442-02 J. H. Hanks (Johns Hopkins University): Dr. Hanks has continued his work on improving the medium for cultivation of *Mycobacterium lepraemurium* (Mlm) as a model of the human infection. Increased Mg^{++} and stable substrates plus unknown compounds provided by yeast extracts and tryptic digests of casein in the medium have enabled him to attain continuous *in vitro* growth of Mlm. Each inoculated Mlm cell gives rise to 20 million new cells during serial transfers to new medium. The *in vitro* growth rates of Mlm were 10 times those in the most susceptible mice. Dr. Hanks is now initiating similar investigations using *Mycobacterium leprae*. The lessons learned from the *in vitro* cultivation of Mlm will be applied to the problem of attaining *in vitro* cultivation of *M. leprae*.

AI 10094-08 W. E. Bullock (University of Kentucky): Dr. Bullock has continued his studies of the immunoregulatory mechanisms in a chronic intracellular infection of rodents caused by *Mycobacterium lepraemurium* (Mlm). He has demonstrated that immunosuppressor activity of considerable potency and complexity is generated during the course of chronic and progressive infection by Mlm. He has now found that leprous spleen cells of Mlm-infected mice appear to produce at least one soluble factor that can partially suppress the immune response of normal spleen cells (non-infected mice).

AI 08214-11 J. H. Peters (Stanford Research Institute): In studies on the metabolic disposition of the chemotherapeutic drug dapsone (DDS) in armadillos, Dr. Peters has determined that, as in man, no differences have been noted between the sexes. Also, DDS disappeared from armadillo plasma at a rate similar to that found in human subjects. It has been determined that plasma levels of DDS at 24 and 48 hours after treatment were approximately one-fourth of the levels observed at these times in man following an oral dose of approximately 0.7 mg/kg. It was concluded that any long-term studies in infected armadillos should contain plans for monitoring plasma levels of antileprosy drugs occasionally, to ascertain that anticipated therapeutic levels of the drugs are obtained in all animals.

AI 08417-09 A. H. Fieldsteel (Stanford Research Institute): Dr. Fieldsteel has continued his studies using the neonatally thymectomized Lewis rat (NTLR) both for the development of chemotherapeutic regimens and as a model of persisting *Mycobacterium leprae*. Several regimens of combination therapy involving dapsone and rifampin were investigated with both goals in mind. The most effective regimens appear to be 10 mg/kg rifampin given by gavage at least twice during administration of the minimal effective dose of dapsone. It is not yet known whether these regimens are sterilizing for the NTLR or whether they result in a persistent state of the infection.

Workshop on the Genetics of Mycobacterium leprae

This was held under the auspices of the U.S.-Japan Cooperative Medical Science Program. Its purpose was to discuss the feasibility of studying the genetics of Mycobacterium leprae using recombinant DNA technology. Various experimental approaches were discussed in terms of scientific and legal feasibility. The conferees recommended that the following approaches to studying the genetics of M. leprae be initiated as soon as possible:

1) The DNA from M. leprae grown in armadillos would be isolated and used to determine the genetic relatedness of M. leprae to other mycobacteria.

2) DNA from M. vaccae (the most likely candidate as a relative of M. leprae among the nonpathogenic, cultivable species) would be introduced into an E. coli K-12 host by means of recombinant DNA technology to determine which mycobacterial gene products are faithfully expressed in E. coli.

It was agreed that, though it will ultimately be desirable to use recombinant DNA methodologies to explore the genetic properties of M. leprae, it is not certain that such experimentation is now permissible under the Recombinant DNA Guidelines (NIH).

Tuberculosis

Tuberculosis, including "atypical tuberculosis," still represents an important infectious disease in the U.S. Although 30,000 new cases of tuberculosis are reported each year in the U.S., this number is small in comparison to the millions of cases found in the developing nations of the world. Tuberculosis in these countries causes an enormous economic loss in terms of health care costs and lost manpower. The tuberculosis program continues to direct its major efforts toward isolation of more specific mycobacterial antigens and the study of basic mechanisms of cell-mediated immunity.

Research Highlights

AI 08401-10 M. B. Goren (National Jewish Hospital): Two classes of mycobacterial glycolipids, cord factor and the sulfatides, have achieved notable prominence as probable virulence factors in Mycobacterium tuberculosis. This may be owing to their individual and synergistic toxic qualities; for the sulfolipids, it may also be because of their powerful antagonism of phagosome-lysosome fusion in cultured macrophages. Dr. Goren has successfully established a macrophage culture system and the behavior of the sulfolipids (SL-I, principal and SL-III, minor) in antagonizing phagosome-lysosome fusion was reconfirmed. In other studies he found that the rate SL-III and the abundantly available SL-I, in combination with Salmonella Re endotoxin, have potent activity in tumor regression in the line 10, strain 2 guinea pig system.

AI 13813-02 H. Gruft (New York State Department of Health): Dr. Gruft is investigating the epidemiology of infection with the atypical mycobacteria. His results have supported the contention that the unusual geographical distribution of persons reacting to atypical mycobacteria (PPD-B) and showing a cross-reaction in the standard tuberculin (PPD-S) test can be explained by

the unique geographical distribution of these microorganisms. They appear to be more prevalent in areas close to a coastal shoreline or in areas with strong prevailing on-shore ocean breezes. Thus, it is established that such non-specific sensitivity to standard tuberculin can be brought about by many members of the atypical mycobacteria. His work is establishing a basis for understanding the epidemiology in relation to the geographical distribution of the atypical mycobacteria.

AI 11807-03 B. M. Sultzer (SUNY Downstate Medical Center): Dr. Sultzer has shown that tuberculin, which is a natural product of the growth of mycobacteria, stimulates the activation of bone marrow derived (B) lymphocytes from nonimmunized mice. This nonspecific form of activation is measured by the synthesis of DNA and the production of antibody to diverse antigens. B lymphocytes from mice immunized with living Bacillus Calmette-Guerin (BCG), an attenuated tubercle bacillus, respond to tuberculin in vitro with enhanced or depressed DNA synthesis depending on the method of infecting the animal with BCG. Thymus-derived (T) lymphocytes from normal or immunized mice, however, are not activated to DNA synthesis when cultured with tuberculin. Dr. Sultzer's objective is to deduce what role these phenomena play in the immunity and hypersensitivity to tuberculosis by determining the mechanisms which are responsible for these responses.

Mycology

Fungi, as infectious microorganisms, have had a major impact on national health through widespread destruction or contamination of food resources as well as etiological agents of disease in humans and animals. Primary fungal pathogens, such as Coccidioides immitis and Histoplasma capsulatum, are well established as significant causes of morbidity and mortality. Also, secondary or opportunistic pathogens (e.g., species of Candida, Cryptococcus and Aspergillus) are being recognized more and more frequently as major causes of both morbidity and mortality in patients with impaired host defense mechanisms. This is a new program initiative still in the developmental stages.

Research Highlights

AI 13336-02 D. Pappagianis (University of California, Davis): Recovery from infection with Coccidioides immitis is generally followed by development of solid immunity against a new infection by this fungus. A killed spherule vaccine was developed, therefore, that provided protection against coccidioidal infection in both a mouse and monkey model system. The dose that could be administered to humans was quite small, relative to the doses needed to immunize mice, because of the irritant activity of the killed spherule vaccine in humans. As a result, conversion of skin test reactivity to coccidioidin following vaccination of humans was irregular. Further studies are now underway to determine if a soluble antigen can be obtained from spherules of C. immitis grown in vitro. This soluble antigen must retain the capacity to immunize, and it must be devoid of the irritant activity of whole killed spherules. Preliminary results suggest that such a protective cell-free antigen may be possible.

AI 13770-02 M. H. Weiner (University of Texas Health Science Center): Systemic aspergillosis and candidiasis are significant and increasing causes of mortality in patients with myeloproliferative diseases and in immunosuppressed transplant recipients. Because of recognized difficulties in antemortem diagnosis of opportunistic mycotic infections, studies to improve immunodiagnosis are clinically highly relevant. Dr. Weiner has developed a radio labeled antigen-binding inhibition assay (RIA) using goat anti-rabbit IgG as a second antibody to detect Aspergillus antigenemia in rabbits with disseminated aspergillosis. Also, a RIA to Candida mannan was developed using goat anti-rabbit IgG as a second antibody. The animal data demonstrate the potential usefulness of antigen immunoassay for diagnosis of fungal disease in man.

AI 14209-01 T. R. Kozel (University of Nevada): The mechanism by which microorganisms evade phagocytosis is a problem basic to understanding many microbial infections. Antiphagocytic surface antigens are found on numerous microbial pathogens. Due to the widespread distribution of antiphagocytic antigens, an investigation of their mechanism of action is fundamental to understanding their role in many infectious diseases. Dr. Kozel is investigating the capsular polysaccharide of Cryptococcus neoformans as an experimental model of an antiphagocytic antigen. He has determined that complement plays no role in phagocytosis of non-encapsulated cryptococci by normal macrophages. Also, IgG appears to be the sole opsonin for phagocytosis of non-encapsulated cryptococci by normal macrophages. Although extensive studies of antiphagocytic surface antigens have been performed previously, the unusual feature of this research is the ability of cryptococcal polysaccharide to adhere to non-encapsulated cryptococci. Experimental variation in polysaccharide concentration allows deposition of a varying amount of capsular material on the yeast surface. Thus, Dr. Kozel is able to investigate a parameter of encapsulation which could not be studied by previous methods using other microorganisms.

Workshop on Medical Mycology Research and Training

The participants in this MIDP-sponsored workshop, representing clinicians and basic scientists from a number of different disciplines, were brought together to consider the status of medical mycology and to focus on two major objectives: 1) To identify reasons for the decline in research support in medical mycology; 2) To recommend supportive measures which could be implemented by NIH. Evaluation of the present status of medical mycology led to the following basic conclusions: 1) There has been a large amount of high quality mycology research, but with only a few species of nonpathogenic fungi. 2) Recent medical literature has reflected an increasing frequency and severity of mycoses. 3) In contrast to the above, the available information indicates serious deficiencies in the number of meritorious investigators in the field of medical mycology. 4) Substantial progress toward dealing with the mycoses as a national health problem would be anticipated only by expanding the training opportunities and research experience in medical mycology.

Virology

Clinical Virology

The goal of this program is to reverse the trend that has separated disease-oriented virologists from molecular virologists and thereby to provide a more stimulating environment to pursue studies on pathogenesis of major viral diseases in man. It is anticipated that such studies will lead to the development of more effective methods for prevention, diagnosis and treatment of viral infections.

Research Highlights

K04 AI 70863-04 P. S. Morahan (Virginia Commonwealth University): Dr. Morahan has focused her research in the area of mechanism(s) of host resistance to viruses and tumors, particularly the venereally transmitted herpes simplex virus type 2 (HSV-2). Using a mouse model for her studies, she demonstrated that cell-mediated immunity (CMI) is the predominant resistance factor to vaginal infection with HSV-2. This CMI resistance is transitory as compared to CMI responses following systemic infections with HSV-2. Also, in systemic infections, activated macrophages play a significant antiviral role, whereas no activated macrophage response was demonstrable following vaginal infection. These studies are significant because they suggest approaches to enhancing resistance to genital herpes in man through use of subunit vaccines to elicit systemic responses to the viral antigens. The systemic responses would then augment the CMI vaginal resistance to produce a longer lasting immunity.

AI 14373-01 J. A. Zaia (Sidney Farber Cancer Institute, Boston): A novel approach was designed to produce large amounts of varicella-zoster immune globulin (V-ZIG) quickly and efficiently. Instead of using blood donors convalescing from recent varicella-zoster (V-Z) infections, plasma from outdated "normal" donor blood was screened for high V-Z virus antibody and positive plasma units were pooled and fractionated for the gamma globulin fraction. The resultant product could not be distinguished from V-ZIG from convalescent donors. Development of this method has assured the availability of sufficient amounts of V-ZIG for the continual passive immunization of lymphoma patients during their first year of tumor therapy.

Persistent Infections and Chronic Viral Diseases

An increasing number of persistent viral infections of man are being recognized for their association with chronic diseases. This program encourages research that will clarify the role of viruses and the responses of the host in chronic and degenerative diseases. Persistent viral infections of animals often provide the best models for studies of pathogenesis. Where possible, cell cultures are employed t' define mechanisms of cell-virus interactions.

Research Highlights

AI 14290-01 M. V. Haspel (Scripps Clinic and Research Foundation): Studies on a mechanism of virus-induced demyelination have been hampered by the lack of a suitable animal model. Mouse hepatitis virus has been known to produce demyelination in some of the survivors following a usually lethal acute

encephalomyelitis. Through the induction of temperature-sensitive mutants, Dr. Haspel has been able to ameliorate the fatal demyelination. With this reproducible model, he is studying the pathogenesis of virus-induced demyelination. In addition, the availability of mutants which differ in ability to produce demyelination suggests the possibility of determining the underlying molecular mechanism of demyelination.

AI 09484-09 M. B. Oldstone (Scripps Clinic and Research Foundation): Lymphocytic choriomeningitis virus (LCMV) produces a persistent infection in mice that eventually causes immune complex disease in the kidneys. Dr. Oldstone and his associates have been studying the structural proteins of the LCMV to determine which are associated with the putative immune complexes. They demonstrated for the first time that all three proteins and specific antibodies to them play a role in the immune complex deposits found in the glomeruli of the kidneys. In addition, it was shown that, in contrast to past speculation, mice infected at birth with LCMV and that became persistently ently infected made antibodies to all the known structural polypeptides.

AI 03771-18 R. L. Crowell (Hahnemann Medical College): Dr. Crowell and associates have developed an enzyme-linked immunosorbent assay (ELISA) for the detection of coxsackievirus antibodies. The test is 100 to 1,000 times more sensitive for detecting group-reactive antibodies among the coxsackie B viruses than either the complement fixation or gel-immunodiffusion assays. The coxsackie viruses cause a large number of very severe forms of human infections for which there presently is no prevention. The ELISA test is a step toward a better recognition of the epidemiology of these viruses and their reported association with chronic myocarditis, pancreatitis, congenital anomalies, muscular dystrophy and other human diseases of unknown etiology.

Symposium on Persistent Viral Infections

In March 1978, a symposium, partially supported by NIAID, was held in Keystone, Colorado to review the state of the art. Included in the review were discussions of recurring herpes infections, congenital cytomegalovirus infections, measles and subacute sclerosing panencephalitis, lymphocytic choriomeningitis, hepatitis B, scrapie, equine infectious anemia, Aleutian disease of mink, and several oncongenic viruses of animals. Some hope for preventing the serious consequences of persistent infections was offered in data that revealed that vaccinees who received live attenuated measles vaccine appeared to be protected against subacute sclerosing panencephalitis, a fatal degenerative sequelae of a persistent infection with wild type measles virus.

Biology of Viruses

If we are to understand how viruses infect man and produce disease, it is essential to know as much as possible about the structure of viruses and how they interact with the cells of the host to replicate and induce immune responses. Through prototype viruses, such as vesicular stomatitis virus (VSV), investigators are focusing on mechanisms of macromolecular synthesis that may ultimately yield vital keys to the process of life itself.

Research Highlights

AI 11112-12 R. R. Wagner (University of Virginia): Dr. Wagner has systemically pursued the molecular biology of vesicular stomatitis virus (VSV) as a prototype rhabdovirus. He and his associates became involved in the first characterizations of RNA, proteins, lipids and carbohydrate components of VSV and, to some extent, their locations within the virus particle. They were also able to determine in their early studies that VSV consists of two separable structural components: the nucleocapsid core and the envelope. Studies on the intracellular biosynthesis and assembly of VSV led to the conclusion that the nucleocapsid is synthesized and assembled separately in the cytoplasm, and that the virus envelope and its two proteins are made and assembled at membrane sites. Synthesis of the two virion components and their assembly are genetically controlled and coded for in the viral genome. Temperature-sensitive mutants are being exploited to study pathogenicity and immunogenicity of VSV. These mutants exhibit differential abilities to switch off cellular protein synthesis and to induce an immune response depending on temperature restriction of transcription and other functions.

AI 11722-05 S. U. Emerson (University of Virginia): The objective of Dr. Emerson's research is to elucidate the steps involved in transcribing and replicating the RNA of VSV. She has demonstrated that the three viral proteins composing the helical nucleocapsid are required for transcription. Now she is defining exactly which catalytic and structural functions each protein performs. These studies are providing insight into how viral RNA synthesis occurs.

AI 10100-07 A. S. Huang (Harvard Medical School): Dr. Huang has continued to use the prototype rhabdovirus VSV in her research. She has demonstrated that VSV contains a virus-associated polymerase which sequentially synthesizes five messenger RNAs by copying one large template. The messenger RNAs are capped with protein at one end and contain polyadenosine at the other. In turn, the RNAs code for structural proteins. The interactions among these proteins and the RNAs are beginning to pave the way toward an understanding of the specific recognition signals and controls existing during macromolecular synthesis. A major interference phenomenon - that mediated by defective interfering (DI) particles - is easily studied in the VSV system. A detailed understanding of this interference may provide a rational basis for the selection of viral vaccines as well as new therapeutic approaches in the treatment of viral infections.

AI 13402-03 D. H. Bishop (University of Alabama): Using techniques that were initially developed in studies of VSV, Dr. Bishop has provided convincing evidence that bunyaviruses and arenaviruses have segmented genomes and the capacity to produce genetic recombinants (reassortment of genetic information). New bunyavirus species have been generated which have been tested for pathogenicity and analysed for genome composition. The ability of either group of viruses to produce recombinants in nature needs to be examined to determine whether recombinant formation can lead to new virus types and new diseases.

AI 12316-04 D. F. Summers (University of Utah): The single envelope glycoprotein of VSV was used as a specific probe for the enzyme glycoslytransferase in fibroblasts from cystic fibrosis (CF) patients as well as a carrier of the genetic defect for CF and a normal individual. The enzyme was found and shown

to be present in all four cell lines. These studies suggest that the basic biochemical defect in cystic fibrosis is not an absence or deficiency in enzymes responsible for the biosynthesis of complex carbohydrate side chains for protein molecules.

Virology Research under the Auspices of the U.S.-Japan Cooperative Medical Science Program

Goals for the Panel in Viral Diseases have been established by a binational agreement between the United States and Japan. These include:

- Basic or applied studies on the control, diagnosis, prophylaxis and epidemiology of rabies;

- Studies on the epidemiology and pathogenesis of dengue and dengue-hemorrhagic fever; and development of vaccines against dengue;

- Studies on the structural elements of arboviruses, including isolation and identification of antigenic components.

Research Highlights

Rabies

AI 09706-08 H. Koprowski (Wistar Institute, Philadelphia): The new WI-38 human diploid cell rabies vaccine (HDCV) developed at the Wistar Institute is produced in France by the Merieux Institute and in West Germany by the Behringwerke AG. It is licensed for pre- and postexposure human prophylaxis in several European and Asian countries; it will soon be licensed in the United States. During the past year, several thousand persons exposed to rabies in Iran, Germany, France and elsewhere were successfully protected with four doses of HDCV plus a booster inoculation given 30 to 90 days later. The HDCV was chosen as the new International Reference Vaccine by the World Health Organization. A new minimum requirement of 2.5 units to be used in an abbreviated schedule (1, 3, 7 and 14 days) was proposed for rabies vaccines. This represents an eightfold increase over the old requirement for duck embryo and brain tissue vaccines. Somatic cell hybrids (hybridomas) between myeloma cells and spleen cells derived from mice immunized with rabies vaccine were found to produce antibodies to rabies virus. Several types of antibodies, including virus-neutralizing antibodies, are present in the supernatant medium of such cultures. Cultures derived from the cloning of a single cell are also positive for antibody production, and antibodies derived from different clones have different specificities. Preliminary evidence indicates that these monoclonal antibodies can differentiate between various strains of rabies virus.

Workshop on the Immunopathology of Rabies

A workshop sponsored jointly by NIAID and the U.S.-Japan CMS Program was held in May 1978 at the annual meeting of the American Society for Microbiology. The one-day workshop was well attended by investigators from various laboratories in the United States and Canada to review the state of the art on immunopathogenesis of rabies and to stimulate ideas for new approaches that

will produce a better understanding of the mechanisms involved. A number of recommendations were offered by the participants for future directions of research. These will be published with a summary of the workshop in the Journal of Infectious Diseases.

Dengue and Other Arthropod-borne Viral Diseases

AI 13380-03 H. G. Haines (University of Miami): Dengue infections continue to be a problem in the Caribbean areas. This is evidenced by the recent outbreaks in Puerto Rico, Jamaica, St. Thomas and Dominica. Dengue type 2 has been isolated from cases in Puerto Rico, while dengue type 1 has been isolated from Jamaica and other areas. This represents the first time that type 1 dengue virus has been isolated in this hemisphere. These investigators have been conducting a seroepidemiologic study of dengue among pregnant women and newborns in the Dominican Republic. They have identified a target population for dengue control, and have accumulated data to indicate that dengue infections are common during the first year of life. These infections cause serious disease in children at this stage of life.

PO1 AI 11132-06 R. E. Shope (Yale University): These investigators have found that dengue virus from a hemorrhagic fever case poorly infects the mosquito Aedes albopictus, the primary dengue vector in rural southeast Asia. The urban vector is Aedes aegypti. The investigators point out that these findings correlate with the urban nature of dengue hemorrhagic fever. Also, they have shown that A. aegypti from the Orient is less competent as a vector of yellow fever virus than strains from Africa or the Caribbean. This observation may help explain why yellow fever does not occur in Asia. Transovarial transmission of yellow fever virus has now been demonstrated in the laboratory. If it can also be demonstrated in nature, it will account for perpetuation of the virus from season to season. There are also serious adverse biohazard implications to those laboratories importing A. aegypti eggs from yellow fever endemic regions into the U.S. for study because they risk importing yellow fever virus as well.

Virology Task Force

A Task Force on Virology was initiated in the fall of 1976 to review the current state of the art and to formulate recommendations for future research. The Task Force has now completed its deliberations and has submitted its report; this is being published in six volumes that will be ready for limited distribution in the fall of 1978. A lay version of the technical report with recommendations is now being prepared for a wider distribution.

MOLECULAR MICROBIOLOGY AND PARASITOLOGY BRANCH

The Molecular Microbiology and Parasitology Branch plans and conducts research grant, program project grant, contract, training grant, fellowship and career award programs in molecular microbiology, biochemistry, genetics, DNA recombinants, physiology, parasitology and medical entomology. It also coordinates the activities of the Parasitic Diseases Panel of the U.S.-Japan Cooperative Medical Science Program.

Approximate Level of Support

Molecular Microbiology Section

Activity	Number	Amount
Research Grants[1/]	162	$10,684,719
Research Contracts	1	337,903
Training Grants	1	109,183
Fellowships	23	259,833
Sub-Total:	187	$11,391,638

1/ Includes 9 Career Awards ($300,067)

Parasitology and Medical Entomology Section

Research Grants[2/]	160	$ 8,927,926
Program Project Grant	1	244,202
Research Contracts	4	254,000
Training Grants	8	489,170
Fellowships	15	172,200
Sub-Total:	188	$10,087,498

2/ Includes 7 Career Awards ($245,513)

Branch	Number	Amount
Research Grants[3/]	322	$19,612,645
Program Project Grant	1	244,202
Research Contracts	5	591,903
Training Grants	9	598,353
Fellowships	38	432,033
Sub-Total:	375	$21,479,136

3/ Includes 16 Career Awards ($545,580)

Molecular Microbiology

During the year, a number of scientific meetings, both domestic and foreign, relevant to the research supported by this Section were attended. Not included in last year's annual report (because it occurred so late in the fiscal year) was the Tenth International Congress of Chemotherapy held in Zurich, Switzerland.

Papers were presented on new antibiotics, synthetic antimicrobials, chemistry, pharmacology and mechanisms of action of antimicrobials, together with bacterial resistance to these agents. The meeting was well attended and productive. In April, 1978 a very stimulating and successful workshop on plasmids and other extrachromosomal genetic elements was held at the Max Planck Institute for Molecular Genetics in West Berlin, Germany. A report on the research papers presented during this meeting has been made previously.

Formerly supported by a research grant, the Stanford Plasmid Reference Center is now supported by the contract mechanism. It will serve as the sole collection and information coordination center of its type in this country, and, as such, is an important establishment which should be very useful to workers in this rapidly expanding area of research.

Early in the year a meeting was arranged here at NIH for those interested in computerized assessment of antibiotic use and antibiotic resistance patterns among the various hospital associated bacterial infections found in American and European hospitals. In attendance were Drs. Curlin, Delappe, Edelman, Horton and Puziss from NIAID; Drs. Winston R. De Monsabert and Richard Norton of the Bureau of Drugs-Food and Drug Administration gave a very interesting presentation. Dr. Robert Edelman will follow up on this with the hope of further involvement of appropriate agencies (NIH, FDA and VA).

The October 17, 1977 issue of the NIH Guide for Grants and Contracts contained a joint announcement by the National Institute of Allergy and Infectious Diseases regarding the availability of a program for funding biological safety facilities grants at the P_3 level of physical containment.

Applications were received, reviewed, and shortly NIAID will fund a number of them. A minimum of 50% of the cost of renovation must be borne by the applicant institution. A major portion of the work carried out in these facilities should involve recombinant DNA technology.

Program Summary

In addition to the basic free-ranging research of relevance to the Institute there are two structured programs supported by this section of the Branch, involving mechanisms of resistance to antimicrobial agents and recombinant DNA molecule research.

(1) Mechanisms of Resistance to Antimicrobial Agents

During the past ten years, the problem of microbial resistance to therapeutic agents has become increasingly apparent. The relevance of this phenomenon to other interests of this Institute such as hospital-associated infections of staphylococcal origin and investigations of the gonococcus is obvious.

At this point, there is substantial clinical and epidemiological evidence that development of this resistance, and especially plasmid (extra-chromosomal) mediated drug resistance, represents a major problem in medical care. Most of the projects are concerned with defining the molecular and biochemical basis for resistance, the principal goal being to elucidate the fundamental biological mechanisms involved in the development of drug resistance by microorganisms. Specific goals involve investigation of the origin, development, evolution, expression and mechanism of drug resistance in a variety of specific microorganisms. Examples of micro-organisms of particular interest are (but not limited to): Entero-bacteriaceae, Pseudomonas, Neisseria, staphylococci, streptococci, mycobacteria, mycoplasmas, and pathogenic fungi.

Research Highlights

AI 10318-06 D. Clewell (University of Michigan): This investigator has observed the presence of a bacterial sex hormone, or pheromone, in Streptococcus faecalis. Homosexual (donor-donor) matings can be greatly enhanced by the addition of this hormone to mating mixtures. The activity of the hormone, which is probably a small protein, is inhibited by chloramphenicol and rifampicin, but not by p-hydroxyphenylazourcil.

AI 10885-06 S. Falkow (University of Washington): Dr. Stanley Falkow has isolated and characterized a structural gene specifying E. coli heat labile enterotoxin. This is of about the same size as the cholera toxin to which it is immunologically related. His data indicate that the E. coli heat labile genes of both animal and human origin are closely related.

AI 10076-11 J. Davies (University of Wisconsin): He has obtained a plasmid from a clinical isolate of Klebsiella pneumoniae which he transferred to E. coli. In addition to resistance to tetracycline, kanamycin, streptomycin and ampicillin, the plasmid conferred resistance to bacteriophages λ and ϕ 21 and E. coli hosts. Resistance to bacteriophage λ was abolished when cells were grown in presence of cyclic AMP.

AI 14997-01 S. Sabet (Eastern Virginia Medical School): Colicins are lethal proteins that bind to and kill sensitive bacteria which contain specific receptors for colicins. A number of colicin receptors have been characterized and shown to be protein components of the cell wall membrane of Escherichia coli. Bacteria that lack the receptors of colicin and those that lost it by mutation do not bind the colicin and are resistant to its killing. A class of mutants termed tolerant are resistant to colicin killing, although they bind the colicin. The tolerant mutants are presumably altered in the mechanism of transmission of the colicin, or its effect, from the receptors of the outer membrane to biochemical targets inside the cell.

Treatment of Escherichia coli K12 with colicin K or E1 caused changes in the protein composition of the bacterial cytoplasmic membrane and an impairment of the membrane-associated ATP-linked transhydrogenase activity. The major compositional changes were loss and/or reduction in the levels of certain protein bands. Colicin K or E1 treatment had no significant effect on the protein composition or the ATP-linked transhydrogenase activity of the cytoplasmic membranes of an isogenic tolerant strain of E. coli K12. The cytoplasmic membranes of the untreated tolerant mutant were characteristically devoid of specific protein bands. It is proposed that protein bands 4 and/or 13 participate in colicin action by acting as receptors for colicins at the cytoplasmic membrane level.

AI 12831-04 D. Storm (University of Illinois): One of the apparent roles of the outer membrane system in gram-negative bacteria is to function as a selective permeability barrier. A number of antibiotics active against gram-positive bacteria are relatively ineffective against gram-negative bacteria presumably because of the implied barrier function of the outer membrane. This interpretation has been strengthened by studies demonstrating synergism between outer membrane perturbing agents such as EDTA or polymyxin B and specific antibiotics. In the case of polymyxin B, it is not totally clear that synergism with other antimicrobials is due to disruption of the outer membrane permeability barrier or to interactions with the inner membrane. In order to resolve this question, polymyxin B was covalently attached to agarose in order to limit interactions with the outer surface of E. coli. These studies demonstrate that immobilized polymyxin B acts synergistically with bacitracin, rifampicin, or lysozyme. It is proposed that synergistic effects exhibited by polymyxin B are due to its interaction

with the outer membrane system.

AI 09423-09 A. Tomasz (Rockefeller University): Using an autolysin-defective mutant pneumococcus as starting material, further mutation(s) were introduced and bacteria with a greatly increased resistance to the bactericidal action of penicillin were isolated. One of these mutants is being characterized. Its cell walls show complete resistance to the hydrolytic action of pneumococcal autolysin; it requires aminoalcohols for growth, but the normal preference of the parental strain for choline is replaced by a preference for ethanolamine. It is suspected that alteration in the chemistry of cell walls is the basis of the defect in the autolytic system of this mutant.

AI 10806-05 D. Tipper (University of Massachusetts): Erythromycin resistance in B. subtilis produces a dramatic alteration in the sporulation process: 100% of the ery^R mutants are temperature sensitive for sporulation. More important to the study of sporulation, however, is the fact that only the sporulation process seems to be altered. These mutants grow vegetatively at 30¼ C and 47¼C with normal generation times, continue protein synthesis at parental levels for several hours following vegetative growth, exhibit normal enzymatic adaptation to the post-exponential metabolic state, and remain viable for some time after vegatative growth ends and after the start of their temperature sensitive period. These facts imply that the temperature sensitivity of sporulation does not result from a general deficiency in protein synthesis due to lack of ribosomal fidelity, stability, or assembly during post-exponential growth. Instead, the sporulation defect seems to be due to a specific deficiency in the expression of sporulation genes. This is reflected in the markedly reduced accumulation of serine esterase activity, and the lack of accumulation of alkaline phosphatase activity in the ery^R spo^{ts} mutants at 47¼ C.

AI 12448-05 J. Davies (University of Wisconsin): The aminoglycoside antibiotics are an important group of therapeutic agents used against bacterial infections; some of these compounds also inhibit eukaryotic protein synthesis in extracts. Radioactive gentamicin is now available and since they have been studying uptake of this drug into bacterial cells, they wished to study the interaction of this drug with its target, the ribosome. First experiments were with E. coli ribosomes and subunits and it was found that gentamicin under a variety of conditions binds to 70S, 50S and 30S particles. By equilibrium dialysis it is possible to detect binding of 4-6 molecules per 70S particle; attempts to analyze this binding by Scatchard plot analysis have been unsuccessful since the multiple binding sites have related affinities of the order of 10^{-7} M. This explains the failure to obtain ribosome mutants resistant to gentamicin, since a single mutation will not eliminate binding as is the case with streptomycin. Many binding experiments have been carried out under different conditions of salt concentration, ribosome concentration, etc., to determine parameters of this binding.

When binding of gentamicin to isolated ribosomal RNA was examined, it was found that only a limited number of sites were available. In fact, the number was small enough to be equivalent to the number of sites on the complete particles; thus it is believed that the aminoglycoside antibiotics of the gentamicin type bind preferentially to the RNA in ribosomes. When yeast ribosomes and ribosomal RNA were examined, a limited number of binding sites were seen, akin to bacterial ribosomes, with a similar binding constant.

(2) Recombinant DNA Molecule Research

During the past few years, the development of certain techniques in the area of molecular biology have made it possible to construct functional DNA molecules *in vitro* which contain segments derived from diverse biological sources. The scientific innovations which led to this technological breakthrough were mostly derivative from basic studies on the mechanism of restriction, which normally acts as a barrier to gene flow among microorganisms, and the molecular biology and genetics of bacterial plasmids, especially those specifying drug resistance. Grantees of this Institute played a preponderant role in the latter development. Whereas DNA recombination in nature has depended on random processes, the experimental techniques now available enable the *in vitro* construction and subsequent replication of DNA molecules needed for specific experimental goals. This basic knowledge coupled with refinements of the existing technology should provide greater knowledge of the mechanisms of pathogenicity (at the molecular level) of viral, bacterial, mycotic and parasitic agents. This information, in turn, may lead to improved prevention, diagnosis and treatment of infectious diseases. Notwithstanding the potential benefits of recombinant DNA molecule research, there may be associated potential biohazards which must be avoided by the design, construction and testing of safer host-vector systems for use in these studies.

The primary goal of this research is the development and utilization of recombinant DNA molecule technology to increase fundamental knowledge and ultimately enhance control of the etiological agents of infectious diseases. Another goal is the synthesis of a variety of biologically useful substances through the construction of bacterial cells containing functional DNA of either plant or animal origin. An equally important goals is the identification, assessment and elimination of any and all potential biohazards encountered in the exploitation of this technology.

Research Highlights

AI 10311-09 D. Dubnau (Public Health Research Institute of N.Y.): During the past several years this laboratory has worked on the molecular basis of recombination using the transformation system of *Bacillus subtilis*. Although convenient for many purposes, the system suffers from the disadvantage that it involves the molecular interaction of a heterogeneous, polydisperse collection of donor DNA molecules with a large, fragile recipient DNA molecule. It was therefore decided to develop a model

system which involves recombination between a small plasmid replicon carrying a cloned segment of genetically marked bacterial DNA, and a homologous unique linear fragment carrying allelic markers. In the process of accomplishing this task they have developed what promises to become a useful and safer alternative to Escherichia coli as a host-vector system for genetic engineering.

Although plasmids do exist in Bacillus, those described to date are cryptic, conferring no selective phenotype on their host. An attempt was made to transfer plasmids derived from Staphylococcus aureus into B. subtilis by genetic transformation. Several plasmids, conferring resistance to kanamycin, chloramphenicol, and streptomycin proved readily transferable to B. subtilis. In the B. subtilis host they replicate and express, achieve the same copy numbers as in S. aureus, and display the same sensitivities to various restriction enzymes as the parent plasmids derived from S. aureus.

AI 14989-01 D. Vapnek (University of Georgia): R538-1 is a conjugative plasmid of compatibility group FII. The plasmid has a molecular weight of 49×10^6 and carries resistance to chloramphenicol, streptomycin and sulfadiazine. By utilizing restriction endonuclease he has constructed a physical map of the plasmid DNA. Molecular cloning techniques have been used to isolate a series of hybrid plasmids carrying EcoR1, HindIII, BamHI and PstI restriction fragments of R538-1. Analysis of these cloned fragments, in conjunction with the detailed restriction map, has provided a fine structure genetic map of the antibiotic resistance genes carried by R538-1.

In vivo transcription and translation of R538-1 DNA has been analyzed by utilizing the physical and genetic maps as well as the cloned fragments. Transcription of individual fragments was determined qualitatively by utilizing the techniques developed by Southern, and quantitatively by carrying out filter hybridization with DNA isolated from the cloned fragments. The results of these experiments demonstrated that most of the R plasmid transcription in strains repressed for conjugal transfer occurs in the region of the R plasmid carrying the antibiotic resistance genes.

Transcription of restriction fragments carrying the genes for Cm^r, Sm^r, Su^r and Hg^r (after induction) has been observed. In plasmids derepressed for conjugal transfer there is a large increase in transcription from the transfer gene region.

To determine the polypeptides coded for by the r-determinant region of R538-1, hybrid ColE1 plasmids carrying restriction fragments from this region were segregated into minicells. Labeling of the minicells with ^{35}S-methionine allowed identification of polypeptides coded for by these fragments. A limited number of polypeptides were identified, with the most active translation occurring from the chloramphenicol transacetylase gene.

The structural gene encoding the catabolic dehydroquinase of Neurospora crassa has been cloned on plasmid pBR322. The dehydroquinase is functionally expressed in E. coli (complementation of an aroD$^-$ auxotroph) and has the physical and immunological properties of the enzyme isolated from crassa . Two restriction fragments carrying the dehydroquinase have been analyzed. These include a 1.9 x 10^6 dalton fragment cloned in the HindIII site of pBR322 (pVK57) and 4.5 x 10^6 dalton fragment cloned in the PstI site (pVK88). Restriction enzyme mapping of these plasmids has demonstrated that the 1.9 x 10^6 dalton fragment is totally contained within the PstI fragment. When the polarity of the HindIII fragment was reversed by cleaving plasmid pVK57 with endo.R.HindIII followed by ligation and retransformation of E. coli, no effect was observed on either the ability of the hybrid to complement an aroD$^-$ auxotroph or on the level of dehydroquinase activity.

The regions of the cloned Neurospora DNA which were transcribed were determined by the techniques developed by Southern (1975, J. Mol. Biol. 98 503-517). The results demonstrated specific regions of transcription within the cloned DNA. Employing RNA-DNA filter hybridization it was shown that about 60% of the transcription occurs from the 1.9 x 10^6 dalton HindIII fragment carrying the dehydroquinase gene.

In addition, the hybrid plasmids carrying Neurospora DNA in both orientations were segregated into minicells. After labeling these minicells with ^{35}S-methionine, a limited number of specific polypeptides were identified. Taken together, these experiments demonstrate that transcriptional and translational starts and stops exist within the cloned Neurospora DNA.

AI 8619-11 S. Cohen (Stanford Medical School): Dr. Cohen studied the expression of Staphylococcus aureus penicillinase plasmid genes in Escherichia coli.

Plasmid p 1258 carries genes necessary for expression of penicillin resistance, arsenate resistance, arsenite resistance, bismuth resistance, lead resistance, cadmium resistance, mercury resistance, erythromycin resistance and plasmid replication and compatibility. EcoR 1 generated fragments of p 1258 DNA were linked to pSC 101, and two different fragments were found to carry sequences able to code for penicillin resistance in E. coli K12.

Using these two plasmids (pSC 112, pSC 113) and some derivatives of them (pSC 122, pSC 142), characteristics of the expression of staphylococcal penicillin resistance genes in E. coli K12 were studied. Simultaneously expression of erythromycin and ion resistances were also investigated. Experiments were performed to analyze the genetic stability of these chimeric plasmids in E. coli K12 and to study the effect of these foreign genes on the ability of E. coli K12 to survive in different conditions.

Parasitology

In June 1977, a workshop on the "immunology of Parasitic Infections" was held in Bethesda, Maryland. A cross-section of immunologists and parasitologists was brought together to discuss current research on immunity and immunopathology in both protozoan and helminth infections. The proceedings of this workshop were published in a 249 page supplement to the November 1977 issue of The American Journal of Tropical Medicine and Hygiene.

The 12th Joint Conference of the U.S.-Japan Parasitic Diseases Panel was held at NIH July 11-13, 1977. Six Japanese and 30 U.S. Scientists presented 33 scientific papers and participated in minisymposia on "New developments in onchocerciasis research" and "Advances in Schistosoma japonicum chemotherapy." The 13th Joint Conference will be held in Okayama City, Japan, October 4-6, 1978.

On December 12-14, 1977 the Branch was represented at the Anglo-American Symposium on Tropical Medicine held at the Royal Society of Medicine in London. One of the major emphases of the discussion was the application of new scientific tools to the solution of long-standing tropical disease problems.

Soon to be issued is a Request for Application for Tropical Disease Research Units program project grants. It is hoped that these units will bring together relevant biomedical knowledge and technology in a multidisciplinary attack on the world's tropical and parasitic diseases.

In view of the increasing clinical and epidemiological evidence that Giardia lamblia is a significant pathogen of man, the Branch will send a representative to a workshop on "Waterborne Transmission of Giardiasis" sponsored by EPA to be held September 18-20, 1978 in Cincinnati. Here the state of the art will be evaluated and research priorities will be discussed. The place of NIAID in implementing these priorities will be a focus of attention in FY 79.

Program Summary

In addition to the basic free-ranging research of relevance to the Institute there are two structured programs supported by this section of the Branch, involving biological regulation of vectors and immunology of parasitic infections.

(1) Biological Regulation of Vectors

This program has as its goal the advancement of fundamental studies which might lead to effective methods of biological regulation of vectors. For the past 30 years, control of pests and disease vectors has been based primarily on the use of synthetic organic compounds which had the "advantages" of long residual action and toxicity to a broad spectrum of target organisms. It has now been shown that because of these very characteristics many of these pesticides are more deleterious than beneficial, when all effects on man and his environment are considered. Furthermore, resistance to broad spectrum

chemical pesticides has reduced their effectiveness in many vector control programs. For these reasons the search for alternative methods of pest control has become imperative, and it is generally agreed that the best approaches will consist of integrated pest management programs which combine biological control, in the broadest sense, with judicious use of more specific chemicals and management of the physical environment. This approach to vector control must be based on adequate information about the ecology of the target organism, the environment in which the control program is to be conducted, effects of control measures on non-target organisms in the environment, and the biology of the disease organisms being transmitted.

Research Highlights

AI 10187-07 K. Nakanishi (Columbia University): With the goal of isolating natural products which exhibit physiological activity in insects, Dr. Nakanishi and his staff have shown that the ground bark of the African medicinal plant Warburgia stuhlmanii yields a highly active insect antifeedant. A 0.1 ppm solution applied to corn leaves will lead to irreversible blocking of the taste sense of African army worms placed on the leaves. The feeding is inhibited to the point of starvation. Another plant being studied has yielded what appears to be a larvicide effective against the important mosquito vector, Aedes aegypti.

AI 12604-04 C. Williams (Harvard University): Using a laboratory developed time-lapse cinematographic technique, Dr. Williams and his colleagues have been studying the response of insect cells to the insect hormone, beta-ecdysone. This technique has enabled them to undertake a frame-by-frame analysis of the hormonally induced differentiation of individual cells and the accompanying changes in cell cycle dynamics. One of the most interesting findings is a 99% decline in the cell's cloning efficiency during its first day of exposure to beta-ecdysone. These studies of ecdysone's action in insects may lead to the discovery of new strategies in the control of insect pests and vectors of disease.

AI 10987-04 R. Silverstein (SUNY): Dr. Silverstein and a collaborating grantee (Sonenshine, AI 10986) have succeeded in isolating and identifying the sex pheromone of the ixodid ticks, Dermacentor variabilis and D. andersoni. The chlorinated phenol 2,6- dichlorophenol was found to be the only compound necessary to elicit the complete series of male behavioral responses culminating in copulation. Experiments are underway attempting to disrupt mating behavior through the incorporation of different concentrations of this pheromone and its analogues in flea collars and dusts.

AI 13295-02 C. Bayne (Oregon State University): Dr. Bayne has been studying minicytes which grow out of snail pericardia in organ culture. He found that minicytes avidly attack and kill schistosome sporocysts. Minicytes are found in greater numbers in snail strains which are refractory to schistosome infection. He feels that minicytes can be considered molluscan analogs of mammalian killer lymphocytes. These studies have formed a solid basis for further work to understand the differences between susceptible and nonsusceptible snails.

AI 08520-11 K. Lie (University of California, S.F.): In this study of snail trematode interactions, with the ultimate goal of finding clues leading to the development of schistosome biocontrol, Dr. Lie has shown that natural resistance in snails may be highly specific, involving not only distinct species but parasite strain differences as well. He hypothesizes that the snail's resistance consists of both humoral and cellular components.

(2) Immunology of Parasitic Infections

The complexity of structure and function of parasites has made the study of immunology of these infectious agents exceptionally challenging and rewarding. Exciting opportunities for the elucidation of mechanisms and manifestations of immunological responses to parasites now exist as the result of the impressive developments in immunology in recent years. Major ultimate goals of studies on immunology of parasitic infections are the development of effective vaccines for the prevention of parasitic diseases (such as malaria, schistosomiasis and filariasis), the intervention in the host response to prevent or ameliorate disease processes which are immunologically mediated, and the development or improvement of immunodiagnostic procedures for parasitic infections, especially as they relate to the immune status of the host.

A related goal of these studies is to contribute to an understanding and solution of basic and clinical problems associated with other disease entities, especially immunological disorders and hypersensitivity states. A number of parasitic infections are excellent models for such studies as: (a) the mechanisms of intracellular immunity, (b) the enhancement or suppression of concurrent infections or tumor development, (c) immunopathological mechanisms, (d) development of disease processes in immunosuppressed or immunostimulated hosts, (e) the biochemical and genetic mechanisms for the development of pathogen variants with different immunological characteristics, (f) the genetic basis for variations in host response, and (g) the role of IgE and other cytotropic antibodies in hypersensitivity.

Research Highlights

AI 13366-03 R.S. Nussenzweig (NYU Medical Center): Sporozoites of several Plasmodium species have been separated from mosquito debris and microbial contents by passing mosquito material through a DEAE-cellulose column. This simple technique has made it possible not only to eliminate most of the contaminants, but to recover rapidly large numbers of viable sporozoites which have retained their infectivity, immunogenicity and capacity to react with known antisera. This technique will be utilized in a long range study of active and passive immunity against malaria.

AI 12065-04 R. Damian (University of Georgia): A potent inhibitor of activated Hageman factor of the intrinsic blood coagulation pathway was identified in S. mansoni. This inhibitor may explain the ability of adult worms to avoid contact activation of the coagulation system in situ in the mesenteric veins. This substance is probably an immunological adaptation

in addition to its obvious role, since Hageman factor is also involved in inflammation pathways. Since this is an adaptation of great physiological importance to the worm, efforts directed toward interference with its inhibitory activity, either immunologically or chemotherapeutically, would be well worth pursuing.

AI 102570-07 L. Ash (UCLA): The presence of immune complexes in the sera of animals infected with filarial worms has often been postulated but never demonstrated. Ash and his colleagues have shown that in gerbil infections antibodies to filarial antigen appear at about the time microfilariae first appear in the blood, rise for several weeks and then decline with time. He also has evidence that intra-vascular thrombosis and inflammation of pulmonary arteries occurs in gerbil infections and is attributable to the action of immune complexes. Preliminary assays with pooled sera have shown the presence of immune complexes at 3 and 12 months infection.

AI 13387-03 P. D'Alesandro (Columbia University): In a comparative study of the antigenic differences between pathogenic and non-pathogenic trypanosomes, Dr. D'Alesandro has shown that the reproduction-inhibiting antibody, ablastin, produced by the non-pathogenic Trypanosoma lewisi is an avid IgG antibody. The relevance of this finding is that it is now technically possible to isolate, purify and characterize its antigen. Such knowledge should provide insights into how parasite reproduction is controlled by the host and may lead to practical methods applicable to the pathogenic trypanosomes.

AI 13126-03 J Mauel (W.H.O): Normal mouse peritoneal macrophages were infected in vivo with Leishmania enriettii, which remained unharmed in such cells for several days. When infected macrophages were exposed to activating stimuli, the parasites were rapidly killed and digested. These activating stimuli included syngeneic lymphocytes stimulated in mixed cultures containing concanavalin A and endotoxin. An increase in the knowledge of mechanisms of immune action is necessary as a basis for the development of vaccines.

(3) General Parasitology Research Highlights

AI 08989-09 W. Trager (Rockefeller Univ.): As an adjunct to his studies of the malaria parasite, Dr. Trager has been interested in determining how the merozoites of Babesia emerge from the host cell. He has discovered within the parasite a coiled organelle which seems to be responsible for making the necessary preparations for merozoite release. His evidence indicates that this organelle contains enzymes which digest and degrade the host membrane which finally breaks down and an opening is made through which the merozoite escapes.

AI 13555-02 R. Lowrie (Tulane): With the goal of laboratory maintenance of the entire life cycle of the filarids - Loa loa, Onchocerca volvulus and several species of Dipetalonema, Dr. Lowrie has been exploring and evaluating the potential of a series of local blood sucking arthropods to serve as intermediate hosts. He has found seven species of the deerfly Chrysops to be capable of supporting complete larval development in Loa loa. This has permitted Dr. Lowrie to directly support and complement other research programs in which infective stage larvae are needed to infect the vertebrate host.

AI 10512-08 H. Saz (Notre Dame): A small particle size formulation of 4- isothiocyanato-4-nitrodiphenylamine (CGP4540) has been found to have high chemotherapeutic activity when administered as a single oral dose to gerbils infected with the filarial worm, Brugia pahangi. This formulation was found previously by Bueding to be highly effective against schistosomes. Saz has also shown the drug to be beneficial against Nippostrongylus and Hymenolepis. To date, no toxic effects have been noted.

AI 07724-12 R. Lewert (University of Chicago): In a field study of a population in an endemic area of schistosomiasis japonica in the Philippines, Dr. Lewert compared several diagnostic tests including the filter paper circumoval precipitin test, the serum circumoval precipitin test and an improved quantitative stool examination technique. His results showed that the filter paper circumoval precipitin test is neither reliable nor sensitive despite its popularity and mass application.

AI 13422-03 I. Sherman (University of California,Riverside): By staining with cationized ferritin followed by electron microscopic observation Dr. Sherman has developed a simple and effective means for differentiating parasite and host cell membranes. He showed that the surface membranes of the parasite are different from one another and from the plasma membrane of the erythrocyte. Employment of these techniques should enable investigators to assess the purity of isolated parasite preparations as well as aiding in the identification of the various membrane systems that are found in the parasite after subcellular fractionation.

(4) Contracts

Transfer of the schistosomiasis supply contract from the University of Michigan to Lowell University Massachusetts was effected smoothly and supply of infected animals has now reached full contract specifications.

The filariasis supply contract at the University of Georgia has continued to be an important factor in the support and stimulation of research on this disease.

The latest reports from the two contractors developing techniques for the cryopreservation of filariae indicate satisfactory progress, especially with the microfilarial stages of Brugia malayi and Dirofilaria corynoides. Recently, microfilariae of W. bancrofti have been successfully frozen, mosquitoes infected and their infective larvae passaged to monkeys.

EXTRAMURAL ACTIVITIES PROGRAM

NIAID

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REPORT OF THE DIRECTOR, EAP

A. Introduction

Fiscal Year 1978 was the first full year of operation under the new federal fiscal year and appropriation schedule. Although it was marked by a difficult start in October with a continuing resolution which did not always continue, it developed into a year during which the reorganization plan for the allocation of Extramural resources was successfully initiated and implemented. The credit for this success is due to the experienced staff of the NIAID/EAP. It was not possible to implement the new data system which is being developed by the Data Systems Planning Committee, however, it will be available for use in Fiscal Year 1979.

Fiscal Year 1978 saw the NIAID scientific review process, utilizing the three Institute chartered committees, operating smoothly. An increased number of new contracts were reviewed by these standing NIAID committees. By the end of the fiscal year, all new contract proposals were being reviewed by the standing committees. The review of the renewal contracts by ad hoc review groups is working well.

During Fiscal Year 1978, the practice of having all "unsolicited" scientific conference proposals; i.e., those arising outside the program areas of NIAID, reviewed centrally by EAP was established. Although there was to be a larger volume of these conference proposals than in the past, the system has screened them well and many meritorious conferences were approved for funding and/or sponsorship.

B. Referral Guidelines and New Referral Handbook

The Office of the Director, Extramural Activities Program, participated with the NIAID Director's Office and NIAID Program Directors in negotiations to resolve and clarify the respective roles of NIAID and other NIH Institutes with regard to research grant referral guidelines. The Division of Research Grants uses these guidelines to decide on the institute assignments for competing research grant applications. Agreements were negotiated to resolve questions involving areas of overlap between NIAID and other institutes. An updated issue of the Referral Handbook of Awarding Organizations of the Public Health Service, reflecting the agreed-upon referral guidelines, was printed by the Division of Research Grants in January 1978.

C. New Guidelines for Requests for Applications (RFAs)

The NIH Office of Extramural Research and Training has developed an issuance concerned with policies and procedures for developing and clearing an RFA. This issuance describes standardized procedures for (1) the development of an RFA by a BID, (2) the technical requirements, (3) the clearance procedures, (4) publication and distribution aspects, (5) guidelines for receipt, referral, and review, and (6) award and management of RFA-initiated grants. These guidelines require that an

RFA must be consistent with the referral guidelines for grant applications as contained in "Referral Handbook". In at least one instance, this requirement has made necessary further negotiations by NIAID with another NIH Institute, since institutes involved with regard to areas of overlap must come to an agreement concerning the institute assignment of applications responding to RFAs.

D. Data Systems Planning Committee (DSPC): Initiation and Progress

The Data Systems Planning Committee (DSPC), made up of NIAID staff, was appointed by Dr. Seal, NIAID Deputy Director, in October 1977 in order to study the problem area of systems for handling NIAID data and to make recommendations to facilitate the obtaining of needed data quickly and accurately. This committee, helped by an additional number of interested NIAID personnel, has made recommendations for new procedures for the computerized handling of NIAID fiscal data which are now operational. In addition, procedures for the coding of projects for program content are being developed which will be consistent with the reorganization of NIAID programs, the new program branch structure, and emerging new subprogram concepts. A procedure for categorizing NIAID-supported projects which are relevant to trans-NIH issues such as Cystic Fibrosis, Kidney Diseases and Nutrition is being developed. Proposed criteria for characterizing NIAID's Programs of Institute Emphasis have been developed, which involve formal acceptance by the NIAID Policy Council, and relate to areas for which some type of Federal intervention is feasible and desirable. A more elaborate and detailed system of coding to designate the specific research area of each active project supported by the Institute has been developed.

E. Clinical Trials

No formal update has thus far been conducted on clinical trials for FY 1977. The word from DRG is that an update of both FY 1977 and FY 1978 clinical trials will be scheduled concurrently in the next few months pending approval of a revised questionnaire by OMB. The program analyst who was responsible for reviewing the applications to identify clinically related projects has resigned. The grants clerk who had been trained to assist the Assistant for Clinical Programs in the clerical and non-technical aspects of such programs, returned to the MIDP. Because no slot was provided to the Program and Project Review Branch for a grants clerk for the AIRC/TIC executive secretary (and it is impossible for an executive secretary of two committees to function without an assistant) the newly hired grants clerk, replacing the one who left, has been trained solely to serve the new executive secretary. Thus, all monitoring, identification, data retrieval and analysis of clinical programs have been reduced to a minimum and will continue to remain so unless adequate help is provided.

F. Personnel

During FY 1978 the EAP had seven staff vacancies occur. A minimum of four months is required to recruit new staff members. Therefore, there was in excess of 28 months (7 vacancies x four months) loss of staff time, or the effective loss of two positions. In FY 1977 the recruitment time average was reported as 2 months.

Retirements

Dr. Roman Kulwich
Mrs. Madeline Rudd

Employees who have left the Institute

Elaine Lee
Virginia Alary
Clarice Dieter
Delores Gibson
Dr. James Ferguson

New Appointments

Dr. Thelma Fisher
Arlene Bailey
Dr. Harley Sheffield
Sue Connor

Training

The Extramural Activities Program continued its interest and active participation in personnel training. During FY '78 the staff attended approximately 40 training programs at a cost of $4,200.

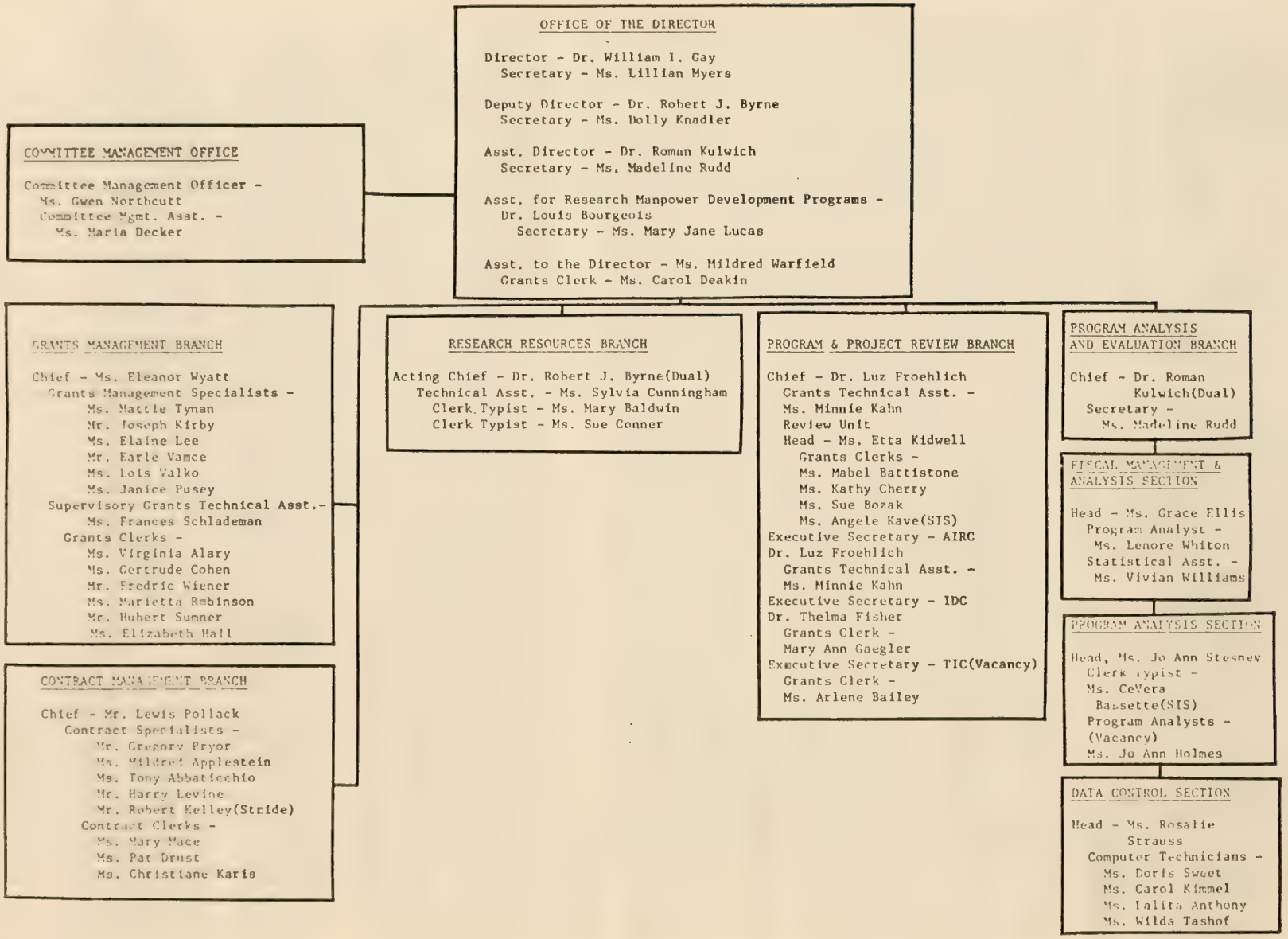
Figure I shows the Organizational Chart.

G. Manpower Development

In keeping with its mission, the National Institute of Allergy and Infectious Diseases supported research manpower development relevant to allergic, immunologic and infectious diseases. This was achieved mainly through the National Research Service Award (NRSA) program.

During the fiscal year 17 T01 (old) training grants were supported with approximately 142 trainees. This was the last year in which these grants received support. It may be that a few of these will be reported in FY 1979 because probable extensions may be granted to those institutions with unexpended funds.

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
EXTRAMURAL ACTIVITIES PROGRAM
ORGANIZATIONAL CHART FOR OPERATING PURPOSES



Effective July 1, 1978

There are 66 training grants being supported under NRSA compared to 49 in FY 1977. Of these there are 30 in the Immunology, Allergic and Immunologic Diseases Program and 36 are in the Microbiology and Infectious Diseases Program.

Table I shows the total number of fellows recommended for support in the NRSA Institutional Training Grants (T-32) for the budget period. These figures do not necessarily reflect the numbers of individuals in training as some of the training grants have only recently been funded and therefore program directors could not recruit fellows. The numbers appointed and in training are shown in Table II.

Table I

	Predoctorals	Postdoctorals
IAID	28	98
MID	45	124
Totals	73	222

Table II

	Predoctorals	Postdoctorals
IAID	18	53
MID	20	84
Totals	38	137

A preliminary report indicates that there are four postdoctoral trainees in parasitology. Three predoctoral and two postdoctoral fellows are receiving training in epidemiology.

H. Decline of Physicians in Research Training

The decline in the number of physicians choosing a research career is of great concern to those in the biomedical sciences. In a recent article published in the New York Times, the Student Loan Marketing Association estimates that half of this fall's freshmen entering private colleges will owe $5,000 when they graduate in 1981 and that figure may triple by 1986. The same article also states that medical school students frequently begin practicing with debts as high as $40,000. While most have an earning level that makes this debt easily manageable over a period of years, educators and others fear that students will not be able to go into such fields as public health or academic medicine. Another important factor that influences the decision not to enter research training is the decrease in Federal research support.

It is suggested that changes be made that would minimize the negative effects of the growing student indebtedness, changes such as A) Lengthen-

ing the 10-year payback schedule of Federal loans, B) Allowing for graduated repayment schedules, C) Consolidating the Federal loan programs into a single program with varying ceilings and repayment schedules that would be sensitive to a borrower's income, D) Providing for reimbursement of tuition indebtedness to those physicians awarded a research training fellowship to prepare them for academic medicine.

I. Freedom of Information Act and Privacy Act

This fiscal year is similar to the last fiscal year in that requests for information under the Freedom of Information Act have been minimal. Requests for Summary Statements under the Privacy Act have maintained the same momentum as previously.

J. Survey of American Society for Microbiology Membership

The survey proposal submitted by the ASM underwent peer review and was approved by the RCAG. The survey instrument together with a statement of justification and the necessary forms have been submitted to the Project Clearance Officer, PHS for approval. When approval has been granted by PHS the material will then be forwarded to OMB for final approval.

K. Committee Management

Charters were renewed last year for all of the Institute committees, including the National Advisory Allergy and Infectious Diseases Council and the Board of Scientific Counselors. As requested, the names of the Institute initial review groups were changed to the Allergy and Clinical Immunology Research Committee, the Microbiology and Infectious Diseases Advisory Committee (with an increase in membership to 15), and the Transplantation Biology and Immunology Committee. The expanded responsibilities for these committees have resulted in a marked increase in their workload.

New members were appointed to the National Advisory Allergy and Infectious Diseases Council in January 1978; however, there are two vacancies resulting from resignations -- one in February 1977, and the other in February 1978. There are vacancies on all of the other committees with the exception of the Transplantation Biology and Immunology Committee. In all cases, nominations have been made for these vacancies, but in most cases the nomination and appointment process has not been completed. Three persons were processed and invited to serve on the Allergy and Clinical Immunology Research Committee, but they declined to serve.

The Department is increasing its efforts to have greater representation of women and minorities on all committees.

L. Conference Proposal Review

The practice of reviewing all "unsolicited" conference proposals, i.e., those arising outside the program areas of NIAID, by the Extramural Activities Program (EAP), was successfully initiated and implemented in Fiscal Year '78. Prospecti were received from the Fogarty International Center as well as directly from the proposers. Those which were accepted for review, primarily on the basis of Institute relevance, were subjected to a primary review by an *ad hoc* scientific group and a secondary review by the RCAG, the latter being scheduled once each quarter. A maximum amount of funds was set aside for the support of these conferences. A total of 63 conference prospecti were received by EAP. Of this number, 53 were reviewed and 36 of these were approved for funding and/or sponsorship for a maximum of $280,334. (See Table III)

Table III

SUMMARY TABLE

CONFERENCE/WORKSHOP PROSPECTI REVIEWED BY NIAID
FY '78

Received..... 63

From Fogarty International Center (FIC)..... 56

Sponsorship & Funds..... 47
Sponsorship Only..... 9

Directly by Institute..... 7

Not Reviewed..... 10

Returned to FIC due to lack of Institute relevance..... 6
Withdrawn by proposer..... 1
Received too late to act..... 1
Re-routed thru FIC due to relevance to other Institutes. 1
Returned to FIC due to Institute policy..... 1

Reviewed..... 53

Approved..... 36

Sponsorship & Funds..... 29
Sponsorship Only..... 7

Disapproved..... 17

Sponsorship & Funds..... 16
Sponsorship Only..... 1

	Number of Conferences	Amount in $
MAXIMUM TOTAL FUNDS APPROVED	29	280,334
Maximum Set-Aside Conference Funds Approved.....	11	76,582
Maximum IAIDP Funds Approved.....	8	90,752
Maximum MIDP Funds Approved.....	9	93,000
Chairman's Grant (Council) Approved...	1	20,000

I. RESEARCH RESOURCES BRANCH

A. Special Activities

ASM Exhibit - The Research Resources Branch handled arrangements for an NIAID exhibit at the American Society for Microbiology Meeting held on May 15-19 in Las Vegas, Nevada. The RRB assumed overall responsibility for the arrangements while manpower used during the exhibit hours was volunteered from the NIAID Extramural Activities Program, Microbiology Infectious Diseases Program and from Intramural Laboratories. The exhibit was well received and gave many visitors knowledge of the various activities being carried out and supported by the Institute. The exhibit itself has been well utilized over the past several years and is now in need of repairs or updating. The NIAID Deputy Director has appointed several institute staff members, including one person of RRB staff, to serve on an NIAID Exhibit Committee. This committee will make recommendations concerning the types of exhibits needed concerning the Institute.

Effective October 1, 1977, the Research Resources Branch (RRB) again was reorganized; the Tissue Transplantation reagents program was transferred from RRB to the Transplantation and Immunology Branch of the Immunology, Allergic and Immunologic Diseases Program (IAIDP). Mr. Donald Hare and Dr. John Ray were transferred to IAIDP. Dr. Robert Byrne assumed the responsibility of Branch Chief with Sylvia Cunningham assuming responsibility for the distribution and cataloging of the remaining microbial and immunological reagents.

The need for well characterized reference reagents as an adjunct to research is well recognized and, in support of this concept, the Research Resources Branch (RRB) conducts a program which distributes a wide range of reagents to research scientists. Reagents for which a critical need exists are identified and arrangements for their production are made by the pertinent Institute Program area. The RRB then arranges for the packaging of the bulk products, catalogs the item and distributes the reagent with the information and technical advice on reagent characteristics and use. The Branch is also sponsoring a contract for the development of a procedure for the processing and packaging of recombinant DNA Vector-Host Systems. In addition, the Branch is assisting with the contract for the establishment of a Plasmid Reference Center.

B. Asthma and Allergic Diseases Program-Allergen Reagents

Final Ampoule testing of the group I, II, and III ryegrass (Lolium perenne) antigens prepared by Worthington Biochemical has now been completed by the certification laboratory (Mayo Clinic). These three antigens are now available for distribution and will be listed and described in the 1978-1980 edition of the Catalog of Research Reagents.

The Investigational New Drug (IND) application for venoms of honey bee, yellow hornets, white-faced hornets, yellow jackets, paper wasps

and hymenoptera venom diluent has been accepted by the BOB/FDA. The venoms and diluent are being employed in a collaborative study among the Asthma and Allergic Disease Centers and other collaborative investigators. The study will compare the efficacy of whole venoms and whole body extracts in the diagnosis of the specific allergies by skin testing. To date venoms have been sent to two (2) investigators for use in the study. One of the investigators has submitted preliminary data which affirms and documents the diagnostic safety, potency and efficacy of the NIH venoms. No systemic reactions or other untoward results occurred.

In addition to the venoms available for distribution under the IND, these venoms are also packaged and labelled for in vitro use also.

The certification laboratory for the allergen reagent program remains at the Mayo Clinic. During Fiscal Year 1978, responsibility for monitoring this contract activity was transferred from the Research Resources Branch to the IAIDP. This central independent facility allows the Institute to quickly verify the correctness of products produced by other contractors. In addition, this laboratory expands the data base for the products beyond that available from producing contractors especially data regarding the biological and in vivo allergenic activities.

Two contract projects (Establishment of an Allergic Dog Colony and Preparation of Polyvalent Ryegrass antiserum) initiated during the latter part of FY 1977 under the sponsorship of the Research Resources Branch were also transferred to the IAIDP for contract monitoring. However, the Chief RRB is serving as project officer on the contract with the University of Pennsylvania (Establishment of an Allergic Dog Colony).

The contract with the Johns Hopkins University for the preparation of polyvalent ryegrass antiserum is proceeding on schedule; it is expected that the bulk product will be available for packaging and labeling by the RRB sometime during the latter part of FY 1978 or the first quarter of FY 1979.

C. Immunological Reagents and Resources

During FY 1978, the contract project requirement for the Maintenance and Breeding of Rabbits of Known Genotype for Use in Immunological Studies was recompeted. Proposals were received from Flow Laboratories and Borriston Research Laboratories. After peer review of the two proposals had been conducted, Borriston Laboratories withdrew their proposal for this contract project. A new contract has now been awarded to Flow Laboratories to continue this project for an additional five year period as they have been doing since 1968. In addition to maintaining the colony of 500 specially bred rabbits for use in NIAID immunological studies, these facilities will provide reference quantities of reagents and limited numbers of rabbits to other responsible investigators doing immunological studies.

The Branch Chief serves as co-project officer for contracts relating to homozygous typing cells and HLA antiserum.

D. Infections

During the latter part of FY 1977 two contracts were awarded and have been progressing during FY 1978. The University of Alabama is performing research and developmental work in various methods of processing and packaging DNA vector-host systems. Preliminary reports indicate that these materials will maintain their viability after being freeze-dried. Stanford University is now under contract to establish a plasmid reference center. This contractor has currently collected approximately 100 strains; however, the required verification of significant characteristics of the plasmids collected has not yet been completed and therefore, no bulk products have been received by the RRB for packaging, storage and distribution. Preparation of the plasmid catalog will not get underway until more data becomes available.

E. Research Resources - Research Reagents

The initial program objectives in this area are nearing completion. Prior years of intensive work have resulted in completed work on the enteroviruses, adenoviruses, rhinoviruses, myxoviruses, and the agents and antigens of hepatitis A and B. In most cases seed virus preparations and corresponding antisera are now available for most of the viruses of public health interest.

The arbovirus certification laboratory located at Yale University has been involved with completing the testing of Bunyamwere and Caraparu immune ascitic fluids. These fluids prepared under another contract have presented a problem to the certification laboratory because of an unknown contaminate. Yale University has therefore, continued this contract at no additional charge in hopes of identifying the contaminate and removing it. To date the contractor has not been successful in these attempts. However, all testing of the 81 arboviruses prepared for this program has now been completed by the certification laboratory.

The contract with the St. Jude Children's Hospital for the preparation and testing of monospecific antisera against all the hemagglutinins, neuraminidases, ribonucleoproteins and the matrix proteins of the influenza viruses of man and animals has been completed during this period. A number of requests for these reagents have already been received and filled. These materials are proving to be a valuable resource in identifying new or unusual serotypes.

The Ohio State University is still serving as the viral reagent testing laboratory. This facility verifies feedback data from recipients concerning storage stability, activity and viability of the various reagents in this program.

After a category of reagents has become established, and the area of research that it supports is well defined, it is of questionable value for RRB to continue storing and distributing that category of reagents for a prolonged period of time. During FY 1975, it was determined that four viral reagent groups fall into this category. RRB, therefore, entered into a five year contractual arrangement with the American Type Culture Collection (ATCC) to transfer the enterovirus, adenovirus, rhinoviruses and arboviruses reagent collections to the ATCC whose prime function is to store and distribute these types of reagents.

Under the contractual arrangement with ATCC, samples of each reagent type will be preserved as a museum function. The viability or homologous antibody activity of a parallel collection of reagents will also be assayed. After each viral group has been assayed and the results reported and reviewed, RRB will discontinue that reagent group from its catalog with a notation that they are available from ATCC and all remaining stock of that group of reagents will be transferred to ATCC for its custodianship. NIH scientists and NIAID contractors will be provided reagents without cost until RRB supply is depleted.

During the first three years of this project, 50 samples of each reagent type for the four viral reagent groups have been transferred to ATCC for the museum function. The assay of the enterovirus, adenovirus and rhinovirus groups has been completed; these three groups have now been transferred to the ATCC. The ATCC will begin to assay the arbovirus reagents during the last quarter of FY 1978.

During this Fiscal Year, the RRB had packaged the 24 HB_SAg-positive serums which were subtyped during the First International Workshop held in Paris in 1975. A panel of these materials is now available for distribution. Announcement of their availability has been made in the Hepatitis Scientific Memorandum.

F. Molecular Anatomy Program

The Molecular Anatomy Program in FY 1978 continued to support the evaluation of the safety and efficacy of prototype monovalent and bivalent HB_SAg vaccines in chimpanzees and human volunteers. At the request of the Project Officer, 1000 doses of an inactivated HB_SAg/adw vaccine was prepared, characterized and given to the BOB, FDA for their use as an interim reference potency standard (BOB Reference Lot 1). For basic studies on the immunochemistry of HB_SAg, improved preparative gel electrophoresis systems were used to isolate the major structural polypeptides of both of the major subtypes. Analysis of the two major polypeptides of an ay subtype by polyacrylamide gel electrophoresis and two-dimensional chromatography peptide-mapping techniques, before and after endoglycosidase treatment, indicated that the larger polypeptide was a glycosylated form of the smaller polypeptide. HBV DNA was isolated and analyzed. The 5' terminal phosphate was removed and the DNA rephosphorylated with γ-32P-ATP. The DNA was also labeled by nick translation using E. coli DNA polymerase I.

Analysis of the products by Hai III restriction nuclease digestion by both approaches indicated there are only two termini in HBV DNA and that they are uniquely located.

Hepatitis A antigen was isolated by zonal centrifugation and externally labeled with ^{125}I to develop a sensitive radioimmunoprecipitation assay for anti-HA. Analysis of purified HAAg polypeptides and nucleic acid indicated hepatitis A should be classified as a picornavirus.

A 50S species or RNA was recovered from RS virus which had been radiolabeled with 3H-uridine and purified by precipitation with concanavalin A and Isopycnic banding in CsCl gradients.

G. Processing and Distribution

In addition to the various program elements detailed above, the Research Resources Branch also distributes coronaviruses, herpes viruses, interferons, mycoplasmas and reoviruses.

The processing contract with the American Type Culture Collection (ATCC) was completed during the Year. Due to the limited number of reagents to be processed at this time, another contract was awarded. Future processing of reagents will be done under a purchase order arrangement.

The Research Resources Branch reagent collection (exclusive of the viral reagents transferred to ATCC) now consists of 690 individual reagents. The repository and distribution contract remains at Flow Laboratories, Rockville, Maryland; a tabular record of distribution by this facility since FY '71 follows:

DISTRIBUTION OF VIRAL MYCOPLASMAL AND ALLERGEN REAGENTS

Fiscal Year	Total Transactions*	Total amps. & vials Distributed
1971	638	12,902
1972	572	21,801
1973	575	19,181
1974	500	9,932
1975	592	6,751
1976	762	10,188
1976(TQ)	192	3,126
1977	613	7,633
1978(3/4 year)	427	5,207

*Does not include the shipments made for testing and packaging purposes and reagents transferred to the American Type Culture Collection.

The following two (2) tables describe the distribution of reagents by groups and also by institutional affiliation during the first three quarters of FY '78.

RESEARCH RESOURCES BRANCH
DISTRIBUTION OF SPECIFIC VIRUS GROUPS
FY 1978 (3/4 year)
OCTOBER 1, 1977 - JUNE 30, 1978

# of Items Avail. (% of Inventory)	Virus Group	SHIPMENTS # of	SHIPMENTS % of	AMPOULES # of	AMPOULES % of # Distributed
9***(1.31)	Adenovirus	9	2.1	62	1.2
17 (2.47)	Allergen	43	10.1	588	11.3
224 (32.61)	*Arbovirus	25	5.9	78	1.5
4 (.58)	Coronavirus	1	.2	1	0
1 (.15)	Cholera	0	0	0	0
20 (2.91)	Recombinant DNA	2	.5	12	.2
20 (2.91)	Enterovirus (Typing Pools)	56	13.1	776	14.9
18 (2.62)	**Hepatitis	36	8.4	1364	26.2
18 (2.62)	Herpes	11	2.6	18	.3
5 (.73)	Interferon	69	16.2	141	2.7
86 (12.52)	Mycoplasma	50	11.7	548	.5
79 (11.50)	Myxovirus Paramyxovirus and related	80	18.7	799	15.4
6 (.87)	Reovirus	6	1.4	29	.6
180 (26.20)	Rhinovirus	9	2.1	182	3.5
	Combinations	30	7.0	609	11.7

Figures based on 427 shipments and 5207 ampoules shipped (excludes testing, packaging and reagents transferred to ATCC).

*Included in this figure are 15 shipments of Arbovirus Grouping fluids totaling 603 ampoules.

**Included in this figure are 2 totalling 1108 shipments to WHO Laboratories totalling 588 ampoules.

***Seed and antisera for adenovirus types 1 thru 31 have been transferred to the ATCC.

RESEARCH RESOURCES BRANCH
FY 1977 SHIPMENTS
OCTOBER 1, 1977 - JUNE 30, 1978

Type of Organization	Shipments to Grantees	Items Shipped	Shipments to Contractors	Items Shipped	Other Shipments	Items Shipped	Total Shipments	Total Items
Universities	28	244	15	68	79	694	122	1006
Commercial	0	0	2	11	23	124	25	135
Hospitals	11	105	3	23	28	259	42	387
Foundations (Non-Profit)	5	37	4	95	18	113	27	245
State or City Health Labs	4	52	0	0	18	199	22	251
Other Federal Agencies	0	0	0	0	16	165	16	165
NIH	0	0	0	0	41	481	41	481
Foreign	0	0	0	0	131	3025	131	3025
WHO	0	0	0	0	0	0	0	0
Reagent Testing	0	0	0	0	21	372	21	372
Bulk for Packaging	0	0	0	0	1	0	1	0
Discard	0	0	0	0	0	0	0	0
ATCC Transfer	0	0	0	0	2	4523	2	4523
Totals	48	438	24	197	378	9955	450	10590

During this year, Branch staff has been updating the 1975-1977 edition of the NIAID Catalog of Research Reagents. Plans are being made to submit the 1978-80 edition to the Government Printing Office for printing during the last quarter of FY '78.

The Branch has continued its efforts to publicize the availability of these valuable reagents to the scientific community. The availability of reagents was incorporated in the NIAID exhibit which was shown at the American Society for Microbiology meeting in Las Vegas, Nevada. The exhibit was again well received and a great deal of interest was expressed, especially by those who were unaware of the NIAID reagents program.

H. Individual Contract and Agreement Tabulation

PROGRAM: IMMUNE SYSTEM AND DISEASE - REAGENTS AND RESOURCES BRANCH: RRB

Principal Investigator Contract Number Contractor FY '78 Obligations	Objectives and Findings
Sullivan, Rudolp R. AI 82565 Flow Laboratories $149,714 (FY 1978)	To maintain a genetically bred rabbit colony of known genotype for use in immunologic investigations. A maximum of 500 adult rabbits will be bred and maintained by expert veterinary care. This is for support of high priority research on genetic control of the immune response and to supply reagents and rabbits to other investigators.

Individual Contract Agreement Tabulation

PROGRAM: INFECTIONS - VIRAL REAGENTS BRANCH: RRB

Principal Investigator Contract Number Contractor FY '78 Obligations	Objectives and Findings
Shope, Robert AI 42517 Yale University Prior Year Funds	This laboratory is the certification facility for the arboviruses. All reagents in this category are assayed in bulk and final packaged form to assure that they meet contract specifications, are potent, and completely characterized. This contract will terminate this year.
Hughes, John R. AI 62513 Ohio State University $17,313	This laboratory serves as a shelf-life viral reagents testing laboratory for members of the enteroviruses, rhinovirus, adenovirus, myxovirus and herpevirus groups. This contract will ensure the integrity of these reference standard reagents by assessing the potency and purity of viral reagents after long term storage.

Individual Contract Agreement Tabulation

PROGRAM: INFECTIONS - PLASMIDS BRANCH: RRB

Principal Investigator Contract Number Contractor FY '78 Obligations	Objectives and Findings
Lederberg, Esther AI 72531 Stanford University $337,903 (FY 1977 funds) Awarded for a 3-year period	The contractor is collecting and coordinating information on bacterial plasmids. During this initial period the contractor has been soliciting strains for the collection assaying a uniform nomenclature to them and verifying their characteristics. Approximately 100 strains have been collected.

Individual Contract Agreement Tabulation

PROGRAM: INFECTIONS - MOLECULAR ANATOMY PROGRAM BRANCH: RRB

Principal Investigator Contract Number Contractor FY '78 Obligations	Objectives and Findings
Gerin, John 00002-07 Energy Research and Development Administration $607,900	The major objective is to develop and apply methods of isolating viruses and viral antigens and the application of those techniques to research on associated diseases. During FY '78 this laboratory was involved in a variety of studies on hepatitis B but the most significant involvement was the evaluation of the safety and efficacy of the prototype monovalent and bivalent HB_sAg vaccines in chimpanzees and human volunteers. Hepatitis A antigen was isolated by zonal centrifugation and externally labeled with ^{125}I to develop a sensitive anti-HA. Analysis of purified HAAg polypeptides and nucleic acid indicated that hepatitis A should be classified as a picornavirus. A 50S species of RNA was recovered from RS virus which had been radio-labeled with ^{3}H-uridine and purified by precipitation with concanavalin A and isopycnic banding in CsCl gradients.

Individual Contract Agreement Tabulation

PROGRAM: INFECTIONS - PROCESSING AND DISTRIBUTION BRANCH: RRB

Principal Investigator Contract Number Contractor FY '78 Obligations	Objectives and Findings
Stanley, Thomas AI 82542 Flow Laboratories $144,678 (MID) $298,362 (IAIDP)	When the reagents program was given the responsibility for production of reference reagents, other responsibilities were also included, i.e., the development and maintenance of appropriate means to receive, process, store and distribute these research materials. The contractor fulfills these responsibilities. During the past year, the contractor has been supplying the storage and shipping services for the RRB, Developmental Applications Branch, the Enteric Diseases Branch and the NCI. As a result of these combined activities, the contractor has made 800 shipments totaling 116,000 vials and 7,000 ml of bulk material. Approximately 30 incoming shipments have been handled totaling 20,000 vials and 1500 ml of bulk material.
Clark-Curtiss, Josephine AI 72533 University of Alabama $171,657 (FY 1977 funds) Contract awarded for a 3-year period	This contractor is determining the optimal processing, packaging and storage conditions for vector-host systems for recombinant DNA molecule research. To date the contractor has investigated various methods of preserving these materials. The methods employed to date include 1) preservation in paraffin - sealed gelatin slabs 2) preservation by freezing in peptone - glycerol, preservation by lyophilization. In addition, a study is being conducted comparing the effects of various storage temperatures. To date, no definite results have been obtained; however, results indicate that these products will sustain viability after undergoing lyophilization.

Donovick, Richard
AI 75-2526
American Type
Culture Collection
$141,240

After a category of reagents has become established and the area of research that it supports is well defined, it is of questionable value for RRB to continue storing and distributing that category of reagents. Therefore, in FY '75, RRB entered into a cooperative enterprise with ATCC to transfer the enterovirus, arbovirus, rhinovirus and adenovirus reagent collections to the ATCC for their custodianship and distribution; complete transfer of the four viral reagent groups will be accomplished over a five-year period. During the first three years of the contract 50 samples of each reagent type for the four viral reagent groups have been transferred for museum storage purposes. Under the terms of the contract each of the four collections of reagents is to be assayed for viability or homologous antibody before the complete transfer is made, therefore, the contractor has been assaying the entero-virus, adenovirus, rhinovirus groups during this initial period. The assay of these groups has been completed, the test results have been reviewed, and the total enterovirus, adenovirus, rhinovirus collections have been transferred to ATCC.

Stevens, David A.
AI 74-2515
American Type
Culture Collection
Prior year funds
Contract is now completed

The program goals of the RRB are to produce, test, package and distribute standardized research reagents to the scientific community. This contract performs all the packing services required for the Branch to accomplish its program goals. During the past year, the contractor processed a total of 42 reagent lots. After the processing is completed, samples of the final product are returned to the certification laboratory to verify that the ampouled contents as identified on the label are correct and to reverify titers. ATCC has been very careful in complying with the contract specifications which stipulate that only one reagent lot should be handled at a time in order to alleviate the possibility of mislabeling.

I. Contract Publications

1. Ziegler, J.L., R.H. Adamson, L.F. Barker, J.F. Fraumeni, J.L. Gerin, and R.H. Purcell. National Institutes of Health International Workshop on Hepatitis B and Liver Cancer. Cancer Research 37: 4672. 1977.

2. Shih, J. W-K., P.L. Tan, and J.L. Gerin. Antigenicity of the major polypeptides of hepatitis B surface antigen. J. Immunol. 120: 520-525. 1978.

3. Gerin, J.L., J. W-K. Shih, V.J. McAuliffe, and R.H. Purcell. Antigens of hepatitis B virus: failure to detect HBeAg on the surfaces of HBsAg forms. J.Gen. Virol. 38: 561-566. 1978.

4. Hoffman, E.J., E.C. Ford, and J.L. Gerin. Isolation of 50S RNA from Respiratory Syncytial virus and ribonucleoprotein. Abstr., Amer. Soc. Microbiol. May, 1978.

5. Shih, J. W-K., G. Hess, P.M. Kaplan, and J.L. Gerin. Polypeptides of Hepatitis B virus (Dane particle). Abstr., Amer. Soc. Microbiol. May, 1978.

6. Gerin, J.L. and J. W-K. Shih. Structure of HBsAg and HBcAg. Proceedings of Second International Symposium on Viral Hepatitis (Eds., G. N. Vyas, R. Schmid, H. Perkins). In Press, 1978.

7. Purcell, R.H. and J.L. Gerin. Hepatitis B Vaccines: A Status Report. Proceedings of Second International Symposium on Viral Hepatitis (Eds., G.N. Vyas, R. Schmid, H. Perkins). In Press, 1978.

8. Feinstone, S.N., Y. Moritsugu, J. W-K. Shih, J.L. Gerin, and R.H. Purcell. Characterization of Hepatitis A Virus. Proceedings of Second International Symposium on Viral Hepatitis. (Eds., G.N. Vyas, R. Schmid, H. Perkins). In Press, 1978.

9. Seeff, L.B., et al. Type B hepatitis after neddlestick exposure and its prevention with hepatitis B immune globulin: final report of the Veterans Administration cooperative study. Ann. Intl. Med. In Press, 1978.

10. Purcell, R.H. and J.L. Gerin: Hepatitis B Vaccines: On the Threshold. Am. J. Clin. Path. In Press, 1978.

11. Hess, G., J. W-K. Shih, J.L. Gerin, R.H. Purcell, and K.H. Meyer zum Bueschenfelde. Hepatitis B antigen: lack of relationship to liver specific protein (LSP). J. Med. Virol. In Press, 1978.

II. PROGRAM AND PROJECT REVIEW BRANCH

A. Introduction

The three review Committees have been renamed to more accurately reflect their review and advisory responsibilities: the Allergy and Immunology Research Committee has been renamed the Allergy and Clinical Immunology Research Committee; the Transplantation Immunology Committee is now called the Transplantation Biology and Immunology Committee, and the Infectious Disease Committee has been renamed the Microbiology and Infectious Diseases Advisory Committee. Whereas the DRG code designations of the two immunology committees have remained the same (AIRC and TIC, respectively), the latter Committee's code designation has been changed to MID (from IDC).

The current NIAID policy is to accept Center applications only in response to an RFA. Although policy is yet unclear concerning Program projects in this regard, applicants of program projects are encouraged to contact program staff before submitting applications. Institute program staff are, however, preparing and issuing RFAs on program projects where renewals are anticipated and are also issuing RFAs on new programs, particularly the MIDP. These require coordination and proper timing so that the time interval between release date of the RFA and the submission date will allow sufficient time for the prospective applicants to prepare applications. Equally important is that the time interval between submission and the Committee meetings should be such that adequate time be allowed for application processing, site visits, the preparation of site visit reports, followed by the full Committee review. The timing of the RFA on the International Centers for Infectious Diseases Research (ICIDR) Program, for example (of which many applications are anticipated), has allowed very little time between submission (January 15) and formal MID Committee review (March 12-13, 1978); as these and program project applications in response to two other RFAs are to be reviewed for the March round, it may not be possible to conduct site visits on these applications.

The AIRC and TIC for the past year have been without a permanent executive secretary. Three scientists from other programs have alternately been detailed to fill the post on a part-time basis, a less than ideal situation. It is hoped that the uneven operation of the two Committees will be corrected when Dr. Harley Sheffield, from the Intramural Programs will come to serve as executive secretary on a permanent basis.

An NIAID Information Brochure on Program Project Grants has been prepared and is undergoing final approval by the Policy Council, pending clarification of the issue of Program Projects and RFAs. This Brochure is primarily for the use of the applicant. It is hoped that the Brochure will offer guidance to the applicant in preparing a complete and well-integrated application.

The release of RFPs is now better timed so that review of new competing or sole source proposals can often be conducted by the full Committee.

B. Allergy and Clinical Immunology Research Committee

The AIRC met three times during the period of November 1977 through September 1978, reviewing three program projects, 15 center applications, six training grant applications, three Allergic Diseases Academic Awards and one sole source contract proposal for a total first year requested amount of $3,883,215. It gave advice to the Institute on such topics as: the need for controlled clinical trials on the evaluation of the Rinkel method of immunotherapy; studies on the use of BPL and the minor determinant mixture in penicillin hypersensitivity; procurement, standardization, and eventual distribution of human IgE and anti-IgE; establishment of a bank of reference sera from patients with hypersensitivity pneumonitis and from hyperimmunized animals; consideration of future contract activity in the procurement, separation, purification, and distribution of various pollen antigens (other than ragweed) and their antisera; what the NIAID role should be in the area of "food allergy."

C. Transplantation Biology and Immunology Committee

The TIC met three times from November 1977 to July 1978 reviewing five program projects, one center grant, two training grants, and 40 contract proposals in response to three RFPs for a total first year requested amount of $4,097,302. An ad hoc contract proposal review was conducted on a requested first year amount of $132,225. It gave advice to the NIAID on possible RFPs on the following: 1) production of homogeneous specific antibody from hybrid cells; 2) procurement and quality control for purified C3; 3) procurement, standardization and distribution of a horse-radish peroxidase reagent for the ELISA test; 4) purified ragweed antigen 5.

D. Microbiology and Infectious Diseases Advisory Committee

The MID met formally three times during the interval of October 1, 1977 to September 30, 1978. In addition, several ad hoc reviews, where certain committee members were also in attendance, were held to review one training grant application and four contract proposals. Additionally, three program project ad hoc review site visits were conducted. The summary statements of these ad hoc reviews went directly to Council. Eight program projects were reviewed by the full Committee, requesting a total amount for year one of $8,926,298. In addition, 31 institutional fellowships were reviewed requesting total funds the first year of $4,218,375. There were 24 contract proposals considered by the full Committee. One unsolicited proposal was disapproved; four were approved. One RFP (78-10, with ten proposals) was decided to be too premature for consideration so that no award was made. Proposals in response to RFP 78-12 were reviewed, but again, a no award decision was made since no new innovative ideas or procedures were presented. First year support requested for contracts considered by the entire MID Committee totaled $2,769,725. Support recommended was negotiated by contract staff members. Twenty-Five renewal contract proposals were reviewed by ad hoc review groups.

The MID Committee was able to meet deadlines throughout the year, but with considerable difficulty, due to inadequate secretarial help. Since the secretarial workload was too large for any one individual to handle, on a 40 hour per week basis, it was necessary to use considerable overtime help. Due to an expanded Committee membership (15 total) and an increased workload, two secretarial or GS 5-6 level individuals are needed desperately in order to carry out duties efficiently, effectively and graciously.

Review Services Unit

The Unit's workload has nearly quadrupled from 1973 to 1978, yet the number of employees have remained virtually the same. The supervisor of the Unit has repeatedly requested, through the Director, EAP, for at least one additional full-time slot, but none has been given. Despite this work overload, processing has proceeded, and application and council books have been prepared and distributed on time.

III. REVIEW AND EVALUATION BRANCH

A. Fiscal Management and Analysis

In keeping with the reorganization of the Institute and the appointment of two program directors to head the IAID and MID programs, a change was implemented in the allocation of funds for grants and contracts in FY 1978 to provide the new directors with funds to carry out their program goals. In order to implement this new plan, support for grants and contracts was distributed between the Director for Extramural Activities and the two program directors.

Assigned to the EAP were most noncompeting regular research grant funds, all competing and noncompeting funds for career, training and fellowship awards, and monies for support of certain predetermined contracts. In addition, a specific sum of money was allocated to the EAP to pay at least 25% of all approval competing traditional research projects on the basis of their priority scores. These were called "Centrally Controlled Funds".

All other research and contract dollars were assigned to the two program directors for funding other research projects, program projects and centers, and the majority of contracts on the basis of programmatic needs.

As a result of these changes, the Fiscal Management and Analysis Section restructured report formats and accounting procedures for grants and contracts to be responsive to the requirements of the new fiscal system. Extramural activities were closely monitored and analyzed to pin point trouble spots and to keep senior staff advised when the need arose for revision of the Institute's master plan.

Due to a modest increase in the NIAID appropriation for research in FY 1978, the Institute was able to keep up with the rising cost of research and still

fund approximately 32% of all approved regular research applications. In addition, over 50% of all approved fellowship, training, career and academic award applications were paid.

Tables IV and V show the final operating budget for grants and contracts as managed by EAP, IAID, and MID in FY 1978.

TABLE IV

NIAID
1978 Operating Plan
Centrally Controlled Funds
(Dollars in thousands)

	IAID		MID		TOTAL	
	No.	Amount	No.	Amount	No.	Amount
COMMITMENT BASE (noncompeting):						
Regular research grants.....	207	19,166	429	30,720	636	49,886
Young investigator grants.....	3	127	4	167	7	294
Career academic awards.....	44	1,544	53	1,867	97	3,411
Individual fellowships.....	25	386	32	490	57	876
Institutional fellowships.....	23	1,471	24	1,992	47	3,463
Old training grants.....	3	240	14	840	17	1,080
Recombinant DNA contracts.....	-	-	5	476	5	476
Molecular anatomy program.....	-	-	1	608	1	608
RRB contracts.....	2	452	3	327	5	779
Escrow against savings.....	-	-	-	-	-	-
Subtotal.....	307	23,386	565	37,487	872	60,873
COMPETING:						
Regular research grants (%)...	99	8,155	197	14,738	296	22,893
Young investigator grants.....	13	547	10	384	23	931
Career academic awards.....	13	446	10	316	23	762
Individual fellowships.....	49	711	60	856	109	1,567
Institutional fellowships.....	7	589	12	723	19	1,312
MARC.....	1	25	-	-	1	25
MBS.....	1	114	-	-	1	114
CDC-nosocomial study.....	-	-	1	500	1	500
Recombinant DNA:.....	-	-	6	412	6	412
Renovation grants- P3.....	(-)	(-)	(5)	(212)	(5)	(212)
New contracts- host & vectors.	(-)	(-)	(1)	(200)	(1)	(200)
Interim facilities.....	(-)	(-)	-	-	-	-
Subtotal	183	10,587	296	17,929	479	28,516
Total, centrally controlled...	490	33,973	861	55,416	1,351	89,389

TABLE V

NIAID
1978 Operating Plan
Program Controlled Funds
(Dollars in thousands)

	IAID		MID		TOTAL	
	No.	Amount	No.	Amount	No.	Amount
COMMITMENT BASE (noncompeting):						
Program projects/centers.....	17	$4,633	8	$3,248	25	$7,881
Regular research grants.....	4	294	5	372	9	666
R&D contracts - Continuations...	24	1,554	55	8,384	79	9,938
Contingency.....	-	122	-	35	-	157
Subtotal.....	45	6,603	68	12,039	113	18,642
COMPETING:						
Program projects/center renewals	8	1,618	3	600	11	2,218
CIRID.....	4	1,011	-	-	4	1,011
Regular research grants.....	12	545	20	1,245	32	1,790
R&D contracts - New.....	4	824	5	2,313	9	3,137
Unscheduled.....	-	1,598	-	909	-	2,507
Subtotal.....	28	5,596	28	5,067	56	10,663
Total, program controlled.....	73	12,199	96	17,106	169	29,305

B. Program Analysis

During fiscal year 1978, the Program Analysis Section answered approximately fifty requests for special analyses and reports in areas of research and research training being supported by the NIAID; about fifty percent of these were non-recurring types of queries. This data was provided to requestors within the NIAID, the NIH and other government agencies, to members of Congress and the public. Areas of reporting included:

1. Programs of special legislative concern, such as Sexually Transmitted Diseases and Tropical Infectious Diseases;

2. Trans-NIH problems, such as cystic fibrosis, kidney disease, blood disorders, diabetes, digestive diseases and broncho-pulmonary disorders;

3. Annual Inventory of Clinical Trials;

4. Scientific management information for those in program planning areas, including "state of the art" and time trend analyses.

Areas of overlap between Programs/Branches resulting from the reorganization of the Extramural Programs have been resolved and all grants and contracts are now assigned according to the new Program/Branch structure. Since October 1977 program representatives and program analysis staff have been involved in designing a more efficient classification system to scientifically categorize NIAID awards data (Data Systems Planning Committee). The Program Analysis Section is in the process of applying this revised and technically detailed system to all projects active and pending in fiscal year 1978.

C. Data Control

The Data Control Section, using the NIH-IBM 370 computer, reports management, budgetary, and programmatic activities for the Extramural Activities Program. In addition, special and repetitive reports are provided for the Immunology, Allergic and Immunologic Diseases Program and the Microbiology and Infectious Diseases Program.

Data Control staff participated, as interested NIAID personnel, in the considerations of the Data System Planning Committee. Development by the Committee of a new scientific information system is in the final stages of completion. As soon as this new system is operational, regular reports as well as the Annual Awards and Projects book will reflect the additional identifications.

In addition, new techniques to establish a tracking system for determining assignment schedules for review of contracts are being developed in order to facilitate the capture and retrieval of this information.

IV. CONTRACT MANAGEMENT BRANCH

The Contract Management Branch provides management services to the Institute's Research Program including solicitation, negotiation, award, and administration of all Institute research contracts. The CMB will continue to implement contract policies and procedures promulgated by higher procurement authority.

There were no staff changes during FY 78.

In accordance with Secretary Califano's initiative to correct major deficiencies in the contracting process, CMB is required to prepare the following quarterly reports:

1. Even distribution of contract awards within the fiscal year plus variances.

2. Procurement requests over $100,000 received by the Procurement Office before the Procurement Plan was signed.

3. Preaward review of proposed contract awards of lesser dollar amounts than those specifically required by procurement regulation 3.50-100.

4. Degree of competition.

One of the principal objectives CMB achieved during FY 78 was to spread the workload more evenly through the fiscal year by shifting contract renewal dates and new contracts as equally as possible in each quarter.

It is estimated that there will be 190 active contracts with a FY 78 allocation of $17,500,000.

V. GRANTS MANAGEMENT BRANCH

Grants Management Branch staff continued to play a key role in the extramural activities of the Institute in fulfilling its responsibilities for the policy review of grant proposals, issuance of awards, management of the business aspects of all grants and awards, proposal inactivations, and closeout of terminated grants and awards. Increased scientific staff involvement in program management of grants and awards had a significant impact on day-to-day operations, especially on the role of the management Specialists who serve as interpretors and advisors to Institute staff on HEW/PHS/NIH policies and procedures pertaining to grants and awards.

A major investment of time and effort was expended in the review and response to proposed and published HEW/PHS/NIH policy and procedural issuances and in developing internal procedures required for grants management and program staff guidance in implementing new and revised policies. Procedural changes, during this reporting period which had the greatest impact on operations were the recombinant DNA research regulations and the change in payment of fellowship stipends from direct payment to Fellows by NIH to payment through the sponsoring institutions.

GMB staff members participated in several management seminars and workshops hosted by representatives of the grantee community. These included:

1. Association of American Medical College-Group of Business Affairs--NIH Seminar, Attended by approximately 50 AAMC-GBA members and 54 NIH representatives. The theme of the 2-1/2 day seminar was "Interaction in Grant Programs," with major emphasis on business administration procedures of mutual interest to both groups.

2. Conference on "NIH Grants Administration," University of California, L.A.

3. Seminar on the "Administration of NIH Grant Programs," Stanford University. Approximately 400 persons representing 30 grantee institutions in the northern and southern California regions attended these two 2-day meetings which provided a unique opportunity for interchange of ideas between NIH senior grants managers and University research administrators, and for discussion of new policy formation and implementation, regulations and procedures, and aspects of grants administration of common concern and interests.

There was increased participation by GMB staff in activities sponsored by the Grants Management Advisory Council. Involvement included:

1. GMAC Workshop - Airlie House Conference Center, Warrenton, Va. Management Specialists participated as co-leaders of modules concerning topics identified by the GMAC membership of special

interest and importance in application review and the management of grants and awards.

2. GMAC Subcommittee to Consider New Payment Procedures for Individual Fellows - Member, E. Wyatt, GMO

3. GMAC Subcommittee to Study Problems Associated with NRSA Paybacks - Member, Mattie Tynan, Specialist

4. GMAC Subcommittee to Survey Training and Continuing Education Needs of the NIH Grants Management Community - Member, Janice Pusey, Specialist.

The Grants Management Officer also participated in a PHS-sponsored 2-day training course in Financial Evaluation of Grant Budget Applications. The program was designed to provide grants management personnel with the fundamentals to effectively perform cost analyses of grant applications, determine financial management capabilities of existing and prospective grantees, and incorporate audit data into the financial evaluation process.

OFFICE OF THE SCIENTIFIC DIRECTOR, NIAID
1978 Annual Report

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SUMMARY OF PROGRAM

Laboratory and Clinical Research, NIAID

October 1, 1977 through September 30, 1978

Office of the Scientific Director

The following pages contain individual reports and summaries from the ten (10) Laboratories that comprise the Intramural component of the National Institute of Allergy and Infectious Diseases. Administrative responsibility for the overall Intramural Research Program resides in the Office of the Scientific Director (OSD). The OSD has a staff of fifty-five (55) who assist the Scientific Director and the various Laboratories in the management, administrative functioning, and animal care of the Intramural Research Program. Also included in the OSD are the DNA Recombinant Unit on the NIH campus and the Immunology Section.

Dr. Kenneth W. Sell was appointed the Scientific Director in September, 1977. This position has been filled by Laboratory Chiefs who served as Acting Scientific Directors during the interim period of over one year. Dr. Sell is dedicated to further upgrading the centralized animal care facilities and services. To attain this goal, the OSD is purchasing new laminar flow equipment to house the animal racks and will purchase compatible racks and caging when necessary, installing automatic watering systems and chilled water units, and remodeling and reorganizing the animal rooms. Contracts for animal breeding and holding services are also being awarded to better utilize the available animal room space. These modifications and improvements will help provide the Intramural investigators with clean, healthy animals with which to perform their experiments.

Dr. John E. Nutter has been assigned to the Intramural Research Program to take administrative responsibility for research involving biohazardous material and the planned National Biohazard Containment Laboratory (NBCL) to be situated at the Frederick Cancer Research Center. This Laboratory will house a resident research program, provide qualified researchers throughout the world with a laboratory to perform experiments with biohazardous materials within the confines of P4 and P3 containment facilities, perform risk assessment studies, and train investigators in the DNA recombinant technique and other high containment techniques. The NBCL, which is expected to be in operation by the end of 1980, will contain eight P3 laboratories and seven P4 laboratories with enclosed gas-tight cabinet systems and glove boxes. The new facility will replace the interim facilities located at the FCRC Building 550, the annex to Building 41 and the mobile containment trailer located near Building 21, both of which are located on the NIH campus.

During the past year, Dr. Malcolm Martin and his colleagues of the DNA Recombinant Unit have continued their investigations of papovaviruses and have focused their efforts in the following area of recombinant DNA studies. In collaboration with Dr. Wallace Rowe (LVD), complete copies of polyoma virus

(PY) DNA were inserted into plasmid DNA vectors at the Eco RI or the Bam HI sites and cloned in an EK2 derivative of E. coli K 12. In each case, PY-plasmid recombinant clones, with the viral DNA inserted in both orientations, were isolated, propagated in liquid culture, and the PY-plasmid chimeric molecules extensively characterized (Israel and Martin). PY DNA was also cloned in E. coli using a lambda-phage vector DNA in a separate series of experiments (Chan and Martin). In addition to monomeric DNA inserts at the RI site in the lambda DNA vector recombinant DNA molecules consisting of a head-to-tail dimer of PY DNA were also obtained which proved to have the interesting property of yielding monomers of viral DNA during propagation in E. coli (Chan and Martin). The biological activity of PY-plasmid and PY-lambda recombinant DNAs as well as bacteria containing such DNA was evaluated in weanling mice and mouse cells in culture. The results of these experiments indicate that PY DNA is not infectious in mouse cells when it exists as a monomeric insert in a prokaryotic vector (Martin, Chan and Israel). Experiments are in progress to evaluate the tumorgenic and/or transforming properties of PY recombinant molecules.

The administrative staff of the OSD has undergone a reorganization in an effort to provide the Laboratories and the OSD with the total range of administrative functions by the respective Administrative Assistants. This will allow the three (3) Administrative Assistants to be involved, on a daily basis, with their respective Laboratories and to be the focal point for providing advice on administrative matters.

An Editorial Office has been established within the OSD. This office is currently staffed by an Editorial Assistant with the plans to recruit an Editor and clerical support. This office, equipped with the IBM Office Systems 6 Word Processing Center, is the central point for finalizing Investigator manuscripts for publication in scientific journals with respect to grammar, sentence structure and conformance to the specific requirements and format of the intended journal. It is anticipated that this office and its equipment will be able to provide valuable support to the OSD staff, as well as the Intramural Investigators.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-A1-00131-11-OSD

PERIOD COVERED

TITLE OF PROJECT (80 characters or less)

Mechanisms of Hypersensitivity in Histocompatible Inbred Guinea Pigs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Sanford H. Stone, Head, Immunology Section, OSD, NIAID

COOPERATING UNITS (if any)

Dr. Cedric S. Raine and Dr. Ute Traugott, Division of Neuropathology, Albert Einstein College of Medicine, New York, N. Y.; Dr. Richard H. Quarles, DMN, NINCDS, Bethesda, Maryland.

LAB/BRANCH

Office of the Scientific Director

SECTION

Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

For some years, we have been using the unique tool of inbred histocompatible guinea pigs for investigation of mechanisms of hypersensitivity for protection against infectious and autoimmune diseases. Advantages lie in the ability to perform viable adoptive transfer of lymphoid cells, and in the capacity to study genetic factors in certain hypersensitivity and autoimmune phenomena. We have concentrated recently on the pathogenesis of an age-dependent, strain-dependent chronic relapsing autoimmune encephalomyelitis which is strongly reminiscent of multiple sclerosis in man, and on the prevention and treatment of this condition. Chronic EAE has been suppressed by appropriate injection of myelin basic protein and the protection monitored by circulating T cell changes as well as by clinical signs and pathological examination.

Subproject I - Comparison of the pathogenesis of acute and chronic encephalomyelitis

Due to the similarities in the symptomologies of the chronic form EAE and multiple sclerosis, the mechanisms of the experimental model as well as those of acute EAE have been further examined from both the cellular and biochemical points of approach.

Using rosetting techniques employing rabbit erythrocytes for guinea pig T cell analysis, it was shown that the percentage of high affinity T cells decreases dramatically during an attack of acute EAE and that this change can be correlated with clinical signs. In addition, sampling of cells infiltrating the CNS (which can be recovered by ultrasonication) determined that coinciding with the decrease in high affinity T cells in the circulation, significantly higher levels of these cells appear within the CNS compartment.

In the chronic model high affinity T cells showed significant decreases during exacerbations and normal values during remissions. When such animals were rechallenged, circulating high affinity T cells again decreased with the development of signs.

The formation of fibrotic bridges between subpial astrocytes into the subarachnoid space and the migration of Schwann cells to the CNS were appraised in chronically demyelinated CNS lesions. Uncommitted Schwann cells were present around remyelinated fibers in nerve root zones between meningeal cells and along blood vessels, implying they migrate via this route to the CNS. The invasion might be aided by glial fibrosis, a process which leads to surface irregularities in the spinal cord and an extensive extracellular space.

The extensive demyelination that occurs in chronic EAE yields products of interest from a diagnostic viewpoint, and the disappearance of myelin and the quantitative and qualitative characterization of its membraneous subfractions continue to be foci of collaboration between our section and that of Quarles et. al.in NINCDS.

Subproject II - Protection against chronic EAE by injection of myelin basic protein in incomplete Freund's adjuvant

Guinea pigs sensitized for induction of chronic relapsing EAE were either left to develop the disease (unsuppressed) or given a series of suppressive injections of myelin basic protein in incomplete adjuvant. All unsuppressed animals developed chronic EAE, and all suppressed guinea pigs remained healthy over a 27 month period. After rechallenge, all unsuppressed animals developed EAE 2-4 weeks later; suppressed animals remained healthy. High affinity circulating T cells in suppressed guinea pigs remained significantly elevated during, and for a short period after, the series of suppressive injections followed by normal values which did not change after rechallenge. This was in contrast to significant decreases in unsuppressed animals with the development of clinical signs. Pathological examination of the CNS complemented

the T-cell and clinical changes. These aspects continue to be investigated in terms of their relevance to the treatment of multiple sclerosis.

Publications:

Raine, C. S. and Stone, S. H.: Animal model for multiple sclerosis. Chronic experimental allergic encephalomyelitis in inbred guinea pigs. N.Y. State J. of Medicine. 77:1693-1696, 1977.

Traugott, U., Stone, S. H. and Raine, C. S.: Experimental allergic encephalomyelitis - migration of early T cells from the circulation into the central nervous system. J. Neurol. Sci. 36:55-61, 1978.

Raine, C. S., Traugott, U. and Stone, S. H.: Suppression of chronic allergic encephalomyelitis: Relevance to multiple sclerosis. Science 201:445-448, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-A1-00132-11-OSD

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Growth Regulation in Microorganisms and in Tissue Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Roy Repaske OSD, NIAID

COOPERATING UNITS (if any)

Dr. T. Theodore, NIAID
Dr. B. Fraser, NIAID

LAB/BRANCH

Office of the Scientific Director

SECTION

Recently assigned to the Unit on Recombinant DNA

INSTITUTE AND LOCATION

NIAID/NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
24/12	1	12/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Carbondioxide was shown to have a regulatory function on cell growth. The immediate effect of CO_2 on growth of E. coli was to initiate RNA and protein synthesis. The optical density of the culture paralleled the increase in protein. Cell division, however, was delayed. On removal of CO_2, RNA synthesis stopped immediately, and after a short delay, protein synthesis decreased to a low rate, while cell division continued to about one doubling. All early products of CO_2 fixation had to be present to replace the CO_2 requirement. Malonate incorporation into cellular fatty acids was analyzed to determine if acetyl carboxylase was the controlling reaction.

Project Description

The objective of this project is (1) to identify the metabolic system in which the participation of CO_2 is required for control of growth and (2) to investigate the mechanism by which this system regulates cell growth.

Methods Employed:

The objectives will be accomplished by using two general approaches simultaneously: (1) physiological growth studies to define the responses and to characterize the CO_2 effect on whole cells, and (2) analytical biochemical studies to identify the enzyme or enzyme system involved to determine the mechanism by which the enzyme or its product function in a regulatory capacity. We have devised a system for growing cultures which accurately establishes and maintains a gas atmosphere containing as many as three gases in any desired proportions; the proportions of gases can be rapidly changed at any time during the experiment. Substituting known CO_2 fixation products for CO_2 or inducing alternate biochemical pathways which circumvent the need for a CO_2 fixation reaction can be used to tentatively identify the growth regulatory system. This will be confirmed by analyzing the cells after pulse labeling experiments. Fatty acids will be identified by mass spectometry. After the identity of the CO_2 regulatory system is confirmed by labeling experiments, conventional biochemical procedures will be used to attempt to determine the interrelationship between this system and those related to cell growth, i.e., initiation of DNA replication and protein synthesis. E. coli growing anaerobically appears to be the best choice for study on theoretical grounds. An E. coli mutant (thy-Ura-Hist-) will be used to follow DNA, RNA, and protein synthesis.

Major Findings:

From the several microorganisms which had been studied, E. coli was selected as a model for analyzing how CO_2 regulated growth. E. coli had the advantage of having an extensive background of information regarding its metabolism, it grows rapidly and shows a CO_2 response in both aerobic and anaerobic modes of growth. Changes in optical density (OD), cell numbers, cell size distribution, total protein and total RNA were examined in relation to the addition and deletion of CO_2 to the culture. None of the growth parameters changed when an inoculum was added to medium without CO_2 in the atmosphere. Immediately after adding CO_2, however, OD, cell size, protein and RNA began increasing at the same exponential rate. The onset of cell division was delayed for about 60 min. Thereafter, cell numbers increased in parallel with the other parameters. When CO_2 was present at the time of inoculation, the same events described above began occurring at zero time.

Deletion of CO_2 from the exponentially growing culture first caused an abrupt cessation of RNA biosynthesis. About 10 to 15 min later, protein synthesis and OD rates fell off to a slower constant rate. Rapid cell division persisted for an hour before leveling off. These experiments showed that RNA biosynthesis was most closely correlated to the presence or absence of CO_2. OD change and net protein synthesis at maximum rates were dependent upon CO_2, but

low levels of protein synthesis and a corresponding OD increase occurred in the absence of CO_2. Cell numbers correlated poorly with CO_2. Cell size distribution confirmed that cells continued to divide in the absence of CO_2 producing populations of small cells. These small cells, typical of stationary phase cells, synthesize RNA and protein and grow in size before cell division occurs as described above.

Supplements of adenine, uracil and orotic acid did not eliminate the requirement for CO_2, and additional supplements of aspartic acid, arginine and glutamic acid, whose syntheses require CO_2, did not relieve the CO_2 requirement. Addition of malonate, a required intermediate for fatty acid synthesis, spared CO_2. Fatty acids area almost exclusively needed for membrane formation. Labeled malonate was incorporated into the lipid fraction of cells (57%) and 68% of that label was found in the fatty acid portion which comprises 2% of the cell. The fatty acids, identified by mass spectrometry, ranged from C_{14} to C_{26}.

Significance to Biomedical Research:

The diversity of cells whose growth is similarly affected by CO_2 (bicarbonate) indicates a fundamental regulatory role for CO_2. The significance of this role is paramount because it controls growth. Unlike other required substrates which cause either unbalanced growth, 'cell aging' or cell death when they are selectively depleted, CO_2 suggests a key metabolic function is controlled which, in turn, controls other systems.

Our results indicate fatty acid biosynthesis may regulate microbial cell growth. Previously, we had shown that bicarbonate concentration affects growth of eucaryotic cells in tissue culture. If an analogeous control system operates in these cells, cell transformation, with its corresponding loss of growth control, may be affecting this regulatory system.

Publications

Repaske, R. and Clayton, M. A. Control of Escherichia Coli Growth by CO_2. J. Bacteriol. In Press (Sept. 1978).

LABORATORY OF CLINICAL INVESTIGATION
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Summary of Program
Laboratory of Clinical Investigation
October 1, 1977 - September 30, 1978

Michael M. Frank, M.D., Chief of Laboratory
and Clinical Director, NIAID

Introduction

The Laboratory of Clinical Investigation has continued the general evolution which began last year when Dr. Michael Frank became Clinical Director and Laboratory Chief. There has been strengthening of some programs but the general format of the laboratory and makeup of its staff has continued along the same lines with major commitments in allergy, clinical immunology and infectious diseases. A number of the members of the Laboratory will be leaving to take other important positions in American medicine. Dr. Allen Kaplan, Head of the Allergic Diseases Section, has resigned to take a position as Chief of Rheumatology, Clinical Immunology and Allergy at the State University of New York at Stony Brook. Dr. Raphael Dolin will be leaving in December to take a position as Head of the Infectious Disease Program at the University of Vermont. Dr. Alan Rosenthal has resigned to take the position as Director of Immunologic Research for Merck, Sharp & Dohme Company. All of these individuals have made major contributions to the success of the Laboratory of Clinical Investigation over the years and all will be missed.

Dr. Michael Kaliner has been named Head of the Allergic Diseases Unit of the Laboratory. Dr. Carl Hammer joined the Laboratory last summer and is making important contributions as a protein chemist and physiologist. Dr. Barton Haynes has joined the Laboratory during this past summer, and will continue as a Senior member of the Clinical Immunology Program. Dr. Stephen Hosea has also been appointed to the senior staff. After one year of additional experience in clinical infectious disease, Dr. Hosea will return to the Laboratory to initiate a program dealing with host defense aspects of bacterial infection. Members of the Laboratory have received a number of honors during the course of the year in recognition of their important clinical and research activities and these are enumerated at the end of this report.

Research Accomplishments

CLINICAL IMMUNOLOGY SECTION

New assays have been developed for the functioning of IgG, Fc receptors and complement receptors in various groups of patients with immunologic disease. All patients with active lupus erythematosus were found to have major abnormalities in their Fc receptors. In contrast, patients with mixed IgM-IgG cryoglobulins, although they had high levels of circulating immune complexes, rarely demonstrated such abnormalities. These patients, of course, do not usually develop renal disease. Patients with Sjögren's syndrome have been divided into two groups, those with Fc receptor clearance defects and those with normal Fc clearance. The patients with normal Fc receptor function have disease which is confined to the glands of the face and head. Patients with abnormalities of Fc receptor function have more widespread illness which includes

rheumatoid arthritis, interstitial nephritis, interstitial pulmonary lung disease and other signs of extra-facial immunologic disorders. The results suggest that this important assay can divide patients with various kinds of immunologic abnormalities into pathophysiologic groups. (Hamburger, Lawley and Frank)

Studies of hereditary angioedema have continued in the Laboratory of Clinical Investigation. Previous studies showed that danazol corrected the genetically controlled protein abnormality in patients with this disease and cured the illness. During this year we studied the toxicity of danazol and the effectiveness of the drug in a large patient population. We determined the minimally effective dose and have defined a new series of toxic side effects of danazol. The drug is still considered relatively non-toxic in this patient population at high risk of death from their disease. In addition, acute attacks of hereditary angioedema, which do not respond to danazol therapy, have been treated with the intravenous infusion of purified C1 esterase inhibitor protein, and this has stopped the attacks within hours of the time of infusion. Thus, the therapy of this condition would appear to be well in hand for most patients with the disease. (Santaella, Hosea and Frank)

New techniques have been developed in the laboratory for the purification of C3, 5, 6, 7, and the C56 complex. New assays have been developed for the C3b inactivator and for the protein B 1H and it has been shown rigorously that both of these proteins are required for C3 inactivation. The particular fragment of C3 which is under study is the fragment responsible for opsonization. It is therefore the fragment responsible for phagocytosis of bacteria, clearance of foreign cells from the circulation, etc. The characteristics of the interaction which leads to the destruction of the opsonically active C3 site have been examined. Studies of the interaction of bacteria with the complement proteins have been conducted and have led to an understanding of the role of complement in the sequestration of bacteria and in the pulmonary localization of bacteria from the circulation. It has been shown that C5a, a fragment of C5, plays a key role in pulmonary localization of bacteria as well as development of the shock lung syndrome in animals with bacterimia. The shock lung syndrome in man will be studied for similar effect. (Gaither, Hammer and Frank)

REGULATION OF IMMUNOLOGIC REACTIVITY

Utilizing a highly sensitive system of polyclonally-induced activation of human peripheral blood B lymphocytes with measurement of single cell antibody production in a hemolysisin-gel plaque forming cell assay, the mechanisms of immunoregulation of B cell function have been studied. Naturally occurring helper and suppressor cell populations responsible for immunoregulation of normal B cell function in man have been identified, and normal human immunological reactivity has been demonstrated to result from a net effect of the balance between these opposing regulatory influences. In addition, the kinetics, mechanisms, and subpopulations involved in mitogen-triggered suppressor cell activity have been delineated. The selective effects of corticosteroids, cytotoxic agents, irradiation, cyclic nucleotides, cell-derived soluble factors, and cell receptor triggering on these regulatory cell subpopulations have been demonstrated. These

studies have led to a greater insight into the mechanisms of immunoregulation of human B cell function in normal immunologic reactivity and in diseases characterized by aberrations of immune regulation. (Fauci, Haynes, Katz, and Dimitriu).

Alterations in proportions, numbers, and functions of immunoregulatory cell subpopulations have been demonstrated in various diseases such as systemic lupus erythematosus, sarcoidosis, and infectious mononucleosis. In addition, aberrations in certain suppressor cell functions with a decrease in suppressor cell activity in lupus, and an increase in suppressor cell activity in sarcoidosis and acute infectious mononucleosis have been demonstrated. These findings contribute to our understanding of the complex mechanisms involved in the primary and/or secondary alterations in immunologic reactivity in these diseases. (Fauci, Haynes, and Katz).

The effect of in vivo corticosteroid administration on human lymphocyte subpopulations has been studied. The previously reported studies on the selective effects of corticosteroids on T lymphocytes have been extended and it has now been demonstrated that corticosteroid administration in man selectively depletes from the circulation one distinct subset of T cells (bearing an Fc receptor for IgM) while sparing another distinct T cell subset (bearing an Fc receptor for IgG). This has important implications in understanding the degree of specificity of certain of the immunosuppressive effects of corticosteroid administration. (Haynes and Fauci).

A large number of patients with the idiopathic hypereosinophilic syndrome are being studied. A therapeutic protocol of corticosteroids and/or hydroxyurea is being employed and highly significant prolongations of survival in this disease with a previously reported extremely poor prognosis have been achieved. In addition, study of this disease has provided an opportunity to characterize various functional capabilities of eosinophils, the interrelationship between immunological abnormalities and hypereosinophilia, as well as the effect of corticosteroids and hydroxyurea on the human eosinophil. (Fauci, Schooley, and Parrillo).

Based on observations related to mechanistic features, clinicopathologic manifestations and therapeutic responses, a revised classification of the entire spectrum of the systemic vasculitides has been proposed in order to better appreciate the distinguishing characteristics as well as the overlap among several of these disorders. In this regard, the previously reported dramatic therapeutic results with the use of cyclophosphamide with or without corticosteroids in Wegener's granulomatosis have continued to be observed. Based on these results, protocols for the use of cyclophosphamide in the polyarteritis nodosa group of systemic necrotizing vasculitides have been initiated and dramatic remissions are being observed (Fauci).

PATHOGENESIS OF DELAYED HYPERSENSITIVITY

The nature of the immunologic defect in chronic mucocutaneous candidiasis has been further defined. Measurements of plasma thymopoietin-like activity yielded essentially normal results and indicated that the lesion was not due to inadequate inductive activity from the thymus. The measurements of LIF production by antigen-stimulated cells suggested that this lymphokine was not essential for

development of delayed cutaneous hypersensitivity responses. Even though lymphocytes from many patients could not produce LIF in response to antigens, in most instances mitogenic stimulation did induce LIF production. This indicates that the cells are genetically capable of producing the lymphokine and suggests that the fundamental defect is either in generation of antigen-responsive cells or failure of antigen recognition at the cell membrane. (Kirkpatrick)

The studies of immunologic reconstitution and resistance to infections continue. The data indicates that both "candida-positive" and "candida-negative" transfer factor has efficacy but that long lasting benefits are more likely when candida-sensitive transfer factor is used. (Kirkpatrick)

INITIATION AND REGULATION OF ANTIGEN RECOGNITION

The interests and proposed course of the Biologic Structure Section is to characterize the role of the macrophage in antigen recognition by T lymphocytes in the expression and initiation of cell-mediated immunity in man and experimental animals. More specifically, this group is interested in exploring the role of the major histocompatibility complex in the genetic regulation of cell-cell interactions required for expression of cell mediated and humoral immunity. The Biologic Structure Section is also actively characterizing the physical state, localization, and fate of macrophage associated antigen. Additional studies of genetics of immune responsiveness to insulin done in man will be assessed and compared to mouse and guinea pig. Determinant selection by macrophages functionally describes Ir gene control of antigen recognition by T lymphocytes in experimental animals. An understanding of the operation of a similar mechanism in man may have implications in the pathophysiology of insulin allergy in diabetics. (Thomas, Schroer, and Rosenthal)

Already, the immunodominant portions of the insulin molecule have been defined in the guinea pig, mouse and man and some of the portions of the molecule which trigger B lymphocytes as opposed to T lymphocytes have also been defined. Various regions of the molecule which trigger suppressor cell activity are also under study.

THE STRUCTURE AND FUNCTION OF KININ MOLECULES

The Allergic Diseases Section has studied the mediators and biochemical pathways that underlie allergic diseases. Proteins required for the generation of bradykinin; namely, Hageman factor, prekallikrein, and high molecular weight (HMW) kininogen are required for surface-dependent initiation of coagulation and fibrinolysis and have been under study. HMW kininogen binds both prekallikrein and factor XI in 1:1 stoichiometric complexes and each Hageman factor substrate is bound to a different kininogen molecule. Thus binding of both prekallikrein and factor XI to surfaces occurs via their interaction with HMW-kininogen. (Thompson, Mandle, and Kaplan).

HMW-kininogen is a single chain molecule from which bradykinin is liberated by two cleavages within a disulfide bridge. The resultant kinin-free kininogen retains coagulant activity that is secondary to the 37,000 dalton light chain. Upon isolation of the light chain by reduction and alkylation followed by gel filtration, the isolated light chain was shown to bind to both prekallikrein and

factor XI and could compete for binding with native HMW-kininogen. The binding constant was of the order of a 10^6 mole^{-1}. (Thompson and Kaplan)

Factor XI was purified and shown to consist of two disulfide linked chains. Upon activation, factor XI consists of an H-chain structure in which identical 50,000 dalton heavy chains are disulfide linked to identical 30,000 dalton light chains. These data suggest that factor XIa has a dimeric structure with two active sites mole. Factor XIa was shown to convert plasminogen to plasmin with the same efficiency as does plasma kallikrein thus the active forms of each Hageman factor substrate mediate the surface-dependent fibrinolysis. (Mandle and Kaplan)

Hageman factor was successfully purified in preparative quantities and was shown to be a 50,000 dalton single-chain molecule which can be cleaved to an active form by kallikrein, plasmin, or factor XIa. Cleavage within a disulfide bridge yields a form that remains attached to the surface and can in turn activate either prekallikrein or factor XI. Cleavage external to the disulfide bond yields Hageman factor fragments which are liberated from the surface and primarily function as a prekallikrein activator. (Miller, Meier, and Kaplan)

CLINICAL STUDIES OF HISTAMINE AND URTICARIA

The ability of histamine to function as a chemotactic factor *in vivo* was studied using skin windows to which solutions were applied over a 24 hour period. Significant selective accumulation of eosinophils was observed in allergic individuals and no correlation with the peripheral eosinophil count was found. Thus allergic individuals appeared to have an intrinsically heightened eosinophil responsiveness and the ability of histamine to function is a chemotactic factor based upon *in vitro* methods was confirmed. (Weinstein, Gallin, and Kaplan)

Cyproheptadine was found to ameliorate the symptoms of cold urticaria in virtually all patients; however, when the wave of histamine release seen with cold challenge was assessed before and after optimal therapy, *no* effect upon histamine release was observed. Thus periactin appears to act by inhibiting the effects of histamine subsequent to release and is consistent with its effect being that of an H-1 receptor histamine antagonist. The fourth described patient with combined cold urticaria and cholinergic urticaria was studied and the kinetics of histamine release followed for each form of challenge. Both disorders were successfully treated with a combination of cyproheptadine and atarax. (Sigler and Kaplan)

STUDIES OF IMMEDIATE HYPERSENSITIVITY IN MAN AND ANIMALS

The immunologic release of the mediators of anaphylaxis from IgE-sensitized human lung tissue is accompanied by increases in cyclic AMP and cyclic GMP; responses attributable to histamine interacting with H-2 and H-1 receptors, respectively. Prostaglandin release is also partly due to histamine stimulation. Mucus glycoprotein secretion from submucous glands and goblet cells is stimulated by mecholyl, histamine and anaphylaxis. Rat mast cells contain both peroxidase and superoxide dismutase in their granules. These granule-associated-enzymes are not released like histamine and provide an additional source of

mediators. Histamine stimulation of rat mast cells fails to cause an increase in mast cell cyclic nucleotides or influence degranulation. Human platelets contain arylsulfatase type IIA and IIB in their secretory granules. Both platelet arylsulfatase and mast cell peroxidase are capable of destroying SRS-A. Human asthmatic subjects have excessive sensitivity to alpha adrenergic and cholinergic stimulation and diminished beta adrenergic responses in comparison with normal controls. Subjects with allergic rhinitis have normal alpha adrenergic responsiveness but the same degree of cholinergic and beta adrenergic dysfunction as the asthmatics. (Kaliner, Henderson, and Shelhamer)

It is intended to extend our studies of asthmatic patients to attempt to define the defects which predispose to asthma and the pathophysiology of asthmatic disease.

MECHANISMS OF PHAGOCYTIC CELL FUNCTION AND CHEMOTAXIS

The mechanism of leukocyte activation by chemotactic factors has been studied using electrophysiology, fluorescent probe, surface charge and ultrastructural techniques. (Seligmann and Gallin)

Studies assessing the mechanism of modulating leukocyte locomotion indicate that limited secretion of specific granules, which accompanies chemotaxis, is associated with increased cell adhesiveness and increased availability of chemoattractant receptors. Vigorous exocytosis is associated with depressed chemotaxis, decreased availability of chemoattractant receptors, chemoattractant hydrolysis by secreted products and markedly increased cell adherence and aggregation. Human pyrogen has been shown to be a potent stimulator of neutrophil exocytosis. (Dinarello, Klempner, and Gallin)

Studies of the two populations of neutrophils we had identified previously indicate that during the neutropenia that follows in vivo endotoxin or hemodialysis, a subpopulation of neutrophils with poorly demonstrable Fc receptors is the predominant neutrophil left in the circulation. Clinical studies assessing the effect of pharmacologic agents on the neutrophil subpopulations in normal subjects, patients with recurrent infection and host defense defects are underway. (Klempner and Gallin)

STUDIES OF VIRAL GASTROINTESTINAL AND RESPIRATORY TRACT INFECTION

A virus particle morphologically similar to but antigenically distinct from the Norwalk agent has been associated with a waterborne outbreak of gastroenteritis, and the disease has been transmitted to normal volunteers. This is the first documented viral agent of gastroenteritis which has a waterborne reservoir. Cell-mediated immune studies of influenza infection and vaccination in man indicate that both antigenically specific and non-specific cytotoxic responses are seen. Patients with systemic lupus lack cytotoxic responses after vaccination and have variable humoral antibody responses. Clinical trials of monovalent and trivalent inactivated vaccines containing influenza A/USSR/90/77 (H1N1) antigens in volunteers resulted in low reactogenicity and high rates of sero-responses. (Mazur and Dolin)

HERPESVIRUS INFECTIONS IN MAN

Rapid assays have been developed for the detection of Epstein-Barr virus *in vitro* which are suitable for clinical use. Patients with infectious mononucleosis lack the ability to suppress outgrowth of EBV infected lymphocytes until convalescence. Systemic administration of adenine arabinoside continues to demonstrate efficacy in treatment of herpes simplex encephalitis, and trials in herpes zoster are nearing their conclusion. Acycloguanosine, a topically administered antiviral compound, is effective in prophylaxis of genital herpesvirus infections in guinea pigs. (Schooley, Mazur, and Dolin)

STUDIES IN MYCOTIC DISEASE

Studies in inbred guinea pigs have found that sensitization with complete Freund's adjuvant releases into the blood a population of thymic-derived lymphocytes which, in the presence of conconavalin A, suppress the proliferative response of autologous lymph node cells to mitogen. (Hernandez and Bennett)

A radioimmunoassay capable of detecting *Candida albicans* polysaccharide at 20 nanograms/ml has been devised. This assay may provide a rapid diagnostic tool for systemic candidiasis. (Bennett)

Additional studies are underway to provide an understanding of basic and applied aspects of various pathogenic fungi including their morphology, taxonomy, pathology, epidemiology, biochemistry, and genetics. Of special interest at the present are the biochemical differences among serotypes of *Cryptococcus neoformans* and the structure of the capsular polysaccharide of serotype *C. neoformans*. (Kwon-Chung)

STUDIES OF PATIENTS WITH PARASITIC DISEASES

The pathogenesis of chronic Chagas' disease could not be related to lymphocyte blastogenic responsiveness to antigens of the causative trypanosome. Some suggestive associations with absence and presence of certain HLA tissue types, however, were found in chronic Chagas' patients. Although specific cell mediated immune responses to parasite antigens in patients with leishmaniasis were demonstrated they must be differentiated from non-specific T-cell stimulation which can occur. Possible auto-immune responses to retinal antigens is being studied in patients with toxoplasmic chorioretinitis.

A high degree of specific IgE response as measured by histamine release from white cells challenged with microfilarial antigens was demonstrated in patients with tropical pulmonary eosinophilia. In contrast, histamine release of white cells from filarial patients with microfilaremia was low. A similar lack of immune responsiveness by lymphocyte transformation in microfilaremic patients seems related to both serum and cellular inhibitory factors. (Ottesen)

Circulating immune complexes that bind complement were demonstrated in patients with actue schistosomiasis. These complexes decreased after treatment or as the infection became chronic. Antibody response in the IgM fraction to a specific schistosome antigen was found to be characteristic of early infections in man. (Lawley and Ottesen)

Administrative, Organizational, and Other Changes

A number of the members of the Laboratory will be leaving to take other important positions in American medicine. Dr. Allen Kaplan, Head of the Allergic Diseases Section, has resigned to take a position as Chief of Rheumatology, Clinical Immunology and Allergy at the State University of New York at Stony Brook. Dr. Raphael Dolin will be leaving in December to take a position as Head of the Infectious Disease Program at the University of Vermont. Dr. Alan Rosenthal has resigned to take the position as Director of Immunologic Research for Merck, Sharp & Dohme Company. All of these individuals have made major contributions to the success of the Laboratory of Clinical Investigation over the years and all will be missed.

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Honors and Awards

Dr. Michael Frank, Clinical Director, was invited to join the Editorial Boards of The Journal of Clinical Investigation and the journal Blood. Dr. Frank recently completed a four-year period as an Associate Editor of The Journal of Immunology. Dr. Frank was elected to fellowship in the American Academy of Allergy, the American Society for Hematology, and in the Association of American Physicians. Dr. Frank was invited to attend the meeting of the World Health Organization in Geneva on the role of the spleen in parasitic diseases. He was also named to the advisory panel of the American Board of Medical Laboratory Immunologists. He has been appointed to the Board of Directors, Foundation for Advanced Education in the Sciences and has been named Chairman of the Medical Board, Clinical Center.

Dr. Anthony Fauci, Deputy Clinical Director, was appointed National Councillor for the American Federation for Clinical Research, as well as being elected to fellowship in the American Academy of Allergy. Dr. Fauci is serving as Chairman of the Allergy and Immunology Committee, Medical Knowledge Self Assessment Program (MKSAP V), American College of Physicians, and on the Advisory Panel of The American Board of Medical Laboratory Immunologists. He acts as an Associate Editor for The Journal of Immunology and serves on the Editorial Board for The Journal of Immunopharmacology.

Dr. John Bennett was invited to chair a symposium at the International Congress of Chemotherapy, held in Zurich, Switzerland, September 18-23, 1977, and was also invited to participate in a symposium at the XII International Congress of Microbiology, to be held in Munich, Germany, September 3-10, 1978. He was a member of the program committee, Chairman of one session and program participant at the International Conference on Histoplasmosis, held in Atlanta, Georgia, in April, 1978.

Dr. Kwon-Chung has been elected as the Division Chairman (1978-1979) of the Medical Mycology Division, American Society for Microbiology. She was invited to lecture at the First International Conference on Histoplasmosis at Atlanta, Georgia, April 13, 1978. She was invited to present a paper at the First International Symposium on Mycetomas held at Barquistimeto, Venezuela, on May 23-27, 1978, and also was invited to participate in the Symposium at the CII International Congress of Microbiology held in Munich, Germany, September 3-10, 1978.

Dr. Gallin and Dr. Dolin were both elected to membership in the American Society for Clinical Investigation. Dr. Gallin chaired a meeting on chemotaxis with Dr. Paul Quie and the proceedings of this meeting were published as a book. Similarly, Dr. Fauci chaired a meeting on human T-cell activation which will appear in book form.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 ZI 00042-13 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Mechanisms of Fever and Host Responsese

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	A.S. Fauci	Head, Clinical Physiology Section	LCI	NIAID
	J.I. Gallin	Senior Investigator	LCI	NIAID
	J.E. Gadek	Medical Officer		
	D.G. Wright	Medical Officer	LCI	NIAID
	J.B. Edelin	Biol. Lab. Tech.	LCI	NIAID
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LAB/BRANCH (Continued below)
Laboratory of Clinical Investigation

SECTION
Clinical Physiology Section

INSTITUTE AND LOCATION
NIAID, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE DURING CURRENT YEAR.

Cooperating Units (Continued):

F.Gardner (Dept. Med., Univ. Texas, Galveston, TX); H.R. Gralnick (Hematology Service, CPD,CC,NIH); M.Perlow (IRP,NINCDS,NIH): and S.Leapman(National Naval Med. Center, Bethesda, MD)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00043-13 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Immunology and Chemotherapy of Systemic Mycoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.E. Bennett	Head, Clinical Mycology Section	LCI NIAID
OTHER:	A.M. Macher	Clinical Associate	LCI NIAID
	A.D. Hernandez	Guest Worker	LCI NIAID

COOPERATING UNITS (if any)
A. Jennings (Clinical Center, NIH)
R. MacGregor (University of Pennsylvania, Philadelphia, PA)

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Mycology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies in inbred guinea pigs have found that sensitization with complete Freund's adjuvant releases into the blood a population of thymic-derived lymphocytes which, in the presence of conconavalin A, suppress the proliferative response of autologous lymph node cells to mitogen.

A radioimmunoassay capable of detecting Candida albicans polysaccharide at 20 nanograms/ml has been devised. This assay may provide a rapid diagnostic tool for systemic candidiasis.

Project Description:

The general goals of the unit are to study host defense in systemic mycoses and to improve diagnosis and treatment of these infections. Current projects concern serologic tests for mycoses and investigation of the mechanism by which delayed hypersensitivity reactions are controlled.

Objectives:

1) Characterize the cell population present in guinea pig peripheral blood which is capable of suppressing lymphocyte transformation by autologous lymph node cells.

2) Determine the immunodominant oligosaccharide in cryptococcal polysaccharide serotypes A, B, C, and D.

3) Devise an assay for candida mannan that will detect nanogram quantities, such that it might be useful in diagnosing systemic candidiasis.

4) Determine whether trimethoprim is synergistic with sulfamethoxazole in vitro against Nocardia species.

5) Investigate mechanism of anemia induced in man by amphotericin B.

Methods Employed:

1) Strain 13 (inbred) guinea pigs were used with or without sensitization with complete Freund's adjuvant. Mononuclear cells were obtained from cardiac blood by hypaque ficoll gradient. Axillary lymph node cells were obtained from the same animals. The two cell populations were cultured alone or together, with or without conconavalin A, pulsed with ^{3}H-thymidine, harvested and counted in a liquid scintillation counter.

2) The constituent monosaccharides of cryptococcal polysaccharide and their α and β methyl pyranoside derivatives were studied for their ability to inhibit the reaction of rabbit antibody with radiolabeled cryptococcal polysaccharide serotypes A, B, C and D. Antigen-antibody binding was measured by the Farr technique.

3) Mannan from Candida albicans serotype A was prepared, tyraminylated and iodinated with ^{125}I. Approximately 0.6 moles tyramine per mole mannan was achieved, with a specific activity of 5×10^{6} cpm/μg and 80% precipitability by specific antibody. This reagent was employed in both solid and liquid phase radioimmunoassay for mannan and in a Farr assay for antimannan antibody. In addition, an enzyme-linked immunoassay for mannan was devised, coating microtiter wells with sheep antimannan, reacting with mannan and detecting mannan by a double antibody technique. Rabbit antimannan was added, washed off and detected by antirabbit globulin conjugated to alkaline

phosphatase.

4) Ten Nocardia isolates were tested for in vitro susceptibility to trimethoprim and sulfamethoxazole, alone and in combinations using the two drugs in three different ratios to one another.

5) Bioassay of erythropoietin was performed in serum and urine of six patients who developed severe anemia solely due to amphotericin B.

Major Findings:

1) Sensitization of strain B guinea pigs with complete Freund's adjuvant resulted in a population of blood mononuclear cells capable of suppressing conconavalin A induced ^{3}H-thymidine incorporation when cultured with lymph node cells. A population of blood mononuclear cells responsible for the suppression was E-rosetting, nonadherent, and sensitive to either mitomucin C or x-irradiation. These properties indicate that thymic derived lymphocytes play an important role in regulating this phenomenon. (Hernandez and Bennett)

2) Hapten inhibition studies identified an immunodominant sugar only in cryptococcal polysaccharides A and D. Glucuronate and methyl-α-D-glucuronopyranoside caused hapten inhibition, being 10^{-5} as effective as unlabeled cryptococcal polysaccharide on a molar ratio. Methyl-β-D-glucuronopyranoside was 10-fold more effective as the α-derivative, indicating that glucuronate in polysaccharide was β-linked. Reduction of terminyl glucuronyl groups to glucose on serotype D polysaccharide caused hapten inhibition by glucuronate to be decreased more than 10-fold, thereby confirming the specificity of the hapten inhibition. Demonstration of an immunodominant glucuronyl group on serotype A and D cryptococcal polysaccharide but not on B or C polysaccharide is in keeping with the taxonomic affinity between serotype A and D, their high degree of cross-reactivity and the previously postulated terminal position of the glucuronyl residue. (Bennett)

3) Both the enzyme-linked and radioimmunoassay proved capable of detecting C. albicans mannan in buffer, the limits of detection being 100 and 20 nanograms per ml, respectively. The assay was much less sensitive to mannan added to normal serum, due to serum antimannan antibody. P-20 titers of antimannan antibody in normal serum ranged from 1:100 to 1:1000. Attempts to free mannan from mannan-antimannan complexes by enzymatic digestion or reducing agents were unsuccessful. Treatment with trichloracetic acid (TCA) and removal of TCA by dialysis did release mannan without damage but diluted the mannan fourfold. (Bennett)

4) The commercially available fixed-dose combination of trimethoprim-sulfamethoxazole was found to contain far too little trimethoprim to provide a synergistic effect with sulfamethoxazole in vitro, suggesting that this combination is not likely to prove useful in nocardiosis. (Bennett and Jennings)

5) Patients with amphotericin B induced anemia did not show appropriate rises in erythropoietin concentration, suggesting that the drug may induce anemia by decreasing renal production of erythropoietin. (MacGregor and Bennett)

Significance to Biomedical Research and the Program of the Institute:

1) In our guinea pig model, sensitization appears to release into the blood a population of T-lymphocytes which act as suppressor cells in the presence of the polyclonal mitogen, conconavalin A. Understanding how delayed hypersensitivity type of responses might be suppressed has important implications for improving therapy of transplant rejection and autoimmune diseases.

2) Immunochemistry of cryptococcal polysaccharide is of interest in unraveling taxonomic relationships and devising radioimmunoassays.

3) Improved methods for detecting C. albicans in serum may lead to better diagnosis of systemic candidiasis, which is the leading cause of death among mycoses.

4) Clinical studies have failed to determine whether trimethoprim contributes to the beneficial effect of trimethoprim-sulfamethoxazole in nocardiosis. This in vitro study suggests that the much higher cost and possibly increased toxicity of the combination is unwarranted.

Proposed Course:

The studies of trimethoprim-sulfamethoxazole, erythropoietin and hapten-inhibition have been concluded. Suppressor cell studies are being continued to investigate the mechanism of suppression. The methods for detecting Candida mannan are now being applied to clinical specimens.

Publications:

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2. Bennett, J.E., Kwon-Chung, K.J. and Theodore, T.S.: Biochemical differences between serotypes of Cryptococcus neoformans. Sabouraudia 16:1-8, 1978.

3. Diasio, R.B., Bennett, J.E. and Myers, C.E.: The mode of action of 5-fluorocytosine. Biochem. Pharm. 27:703-707, 1978.

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man. Antimicrob. Agents Chemother. 13:271-276, 1978.

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6. Bennett, J.E. and Jennings, A.J.: Factors influencing susceptibility of Nocardia species to trimethoprim-sulfamethoxazole. Antimicrob. Agents Chemother. 13:624-627, 1978.

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9. Kwon-Chung, K.J. and Bennett, J.E.: Distribution of α and a mating types of Cryptococcus neoformans among natural and clinical isolates. Amer. J. Epidemiol. In press.

10. MacGregor, R.R., Bennett, J.E. and Erslev, A.A.: Erythropoietin concentration in amphotericin B-induced anemia. Antimicrob. Agents Chemother. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00045-10 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies on the Interaction of Antibody and Complement on the Production of Immune Damage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immumology Section and Chief	LCI NIAID
OTHER:	C. Hammer	Senior Staff Fellow	LCI NIAID
	T. Gaither	Resident Biologist	LCI NIAID
	R. Reinhart	BLT (Biochemistry)	LCI NIAID
	L. Renfer	Chemist	LCI NIAID
	M. Santaella	Guest Researcher on IPA	LCI NIAID

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

New techniques have been developed in the laboratory for the purification of C3, 5, 6, 7, and C56 complex. New assays have been developed for the C3b inactivator and for the protein Beta 1H and has been shown rigorously that both of these proteins are required for C3 inactivation. The characteristics of this interaction which leads to the destruction of the opsonically active C3 site have been examined. Studies of the interaction of bacteria with the complement proteins have led to an understanding of the role of complement in the sequestration of bacteria and in the pulmonary localization of bacteria. It has been shown the C5a plays a key role in pulmonary localization of bacteria as well as in the development of the shock lung syndrome in animals with bacteriemia. The shock lung syndrome in man will be studied for similar effect.

Project Description:

Objective:

The objective of this program is the long-term study of the role of antibody and complement in the production of immune injury in vitro and in vivo. To this end, major changes have taken place in the investigative thrust of the Clinical Immunology Section within the last year. A new and pronounced emphasis upon protein purification and studies of protein interaction has begun within the laboratory and is already yielding important new results.

Methods Employed:

In all of these studies, the methods employed involved purification of complement components, their use in the sequential build-up of complement intermediates on cells, the identification of sera of patients with certain complement abnormalities so that the sera can be used as reagents, and the purification of immunoglobulins of various classes by standard techniques. Methods for the titration of complement components have been developed in our laboratory as well as in other laboratories, and these are in routine use. Methods for cultivation of various types of bacteria as well as for bactericidal assays have also been developed as have methods for radiolabeling of bacteria. Disc gel polyacrylamide electrophoresis as well as electrofocusing techniques have been developed in the laboratory and methods have been developed to prepare proteins of very high purity. Also large batch methods have been developed for the first time for the purification of the various complement proteins.

Major Findings:

First, techniques have now been developed within the laboratories for the purification of large quantities of C3, 5, 6, and 7 and initial studies have been undertaken for the preparation and purification of the so-called C56 complex which is capable of attaching to, and damaging, unsensitized cells and membranes. Entirely new methodology has had to be developed to prepare a number of these reagents and a major thrust of the laboratory in the last year has been in this very new direction. Already major findings are beginning to appear from this general approach. New methods have been developed for the study of Beta 1H and the C3 inactivator which allows us to examine the effect of these proteins on the opsonically important fragment of C3 C3b. A new radioimmunoassay for C3 inactivator activity has been developed. It has been shown on erythrocyte surfaces as well as in the fluid phase that the C3 inactivator requires the presence of Beat 1H for its function. The kinetics of this interaction as well as the temperature dependence of the interaction has been examined. It has been shown that the degradation of C3 and the release of C3 from the red cell surface requires three separate steps. In the first step Beta 1H interacts with the C3b bound to the erythrocyte surface. This does not lead to loss in C3b function. In the second step, C3 inactivator interacts with this complex of Beta 1H and C3b.

This leads to loss of immune adherence activity but not release of radioactive C3b into the supernatant surrounding the cell. In the third step a trypsin-like enzyme cleaves the C3b which has been prepared for its release by the action of Beta 1H and the C3b inactivator. This release of the radioactive C3b is a sensitive index of a function of this system. It is impossible to develop this assay as a routine procedure and show that one can use it to assay C3 inactivator Beta 1H or the trypsin-like enzyme in serum. All of these points are undergoing further evaluation at the present time.

As part of this program under Dr. Hosea, we have also begun the careful evaluation of the interaction of bacteria with the complement's proteins. A series of potentially clinically very important findings have come from these studies. In the first place it has been shown that in the guinea pig the role of antibody to pneumococci in the removal of the organisms from the guinea pig circulation is limited to activation of the complement system. Antibody, per se, does not contribute greatly to clearance of the organisms. The primary pathway of activation of complement responsible for clearance of the organism is the alternative pathway. The classical pathway is of secondary importance. It appears that when bacteria are injected into the circulation in both guinea pigs and mice, about 20% of the injected organisms are localized in the lung. This pulmonary sequestration of bacteria is never seen with antibody or complement sensitized red cells and is absolutely dependent upon activation of the complement system. In C5 deficient mice, no pulmonary sequestration is noted. The sequestration is not dependent upon any component of complement present on the bacterial surface, but depends absolutely on the activation of the complement system and the generation of the anaphylatoxic fragment of the C5 C5a. It has been shown this year that C5a in a highly purified form causes the pulmonary capillary bed to leak protein and also causes the pulmonary sequestration of polymorphonuclear neutrophils. These two events occur along with the bacterial localization in the lungs. We have postulated that these two events are associated with the development of the shock lung syndrome in man, which is severe pulmonary distress and shock associated with septicemia.

Proposed Course:

We plan to extend our findings of the protein interactions in the complement system and also our findings with regard to the role of bacteria and complement and their interaction. These findings cut right to the heart of an understanding of the pathophysiology of bacterial infection and represent a rather new approach to the study of these kinds of infection. As anti-complementary agents become available it is hoped to apply these agents to the test system under study to determine whether ongoing patients with complement mediated tissue damage can be treated with these agents.

Publications:

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5. Gaither, T.A. and Frank, M.M.: Complement in clinical pathology. In Henry, J.B. (Ed.): Clinical Diagnosis and Management by Laboratory Methods. Philadelphia, W.B. Saunders Co., 16th Ed. In press.

6. Macher, A.M., Bennett, J.E., Gadek, J.E., and Frank, M.M.: Complement depletion in cryptococcal sepsis. J. Immunol. 120:1686-1690, 1978.

7. Frank, M.M. and Hosea, S.W.: Complement. In Cohen, A.S. (Ed.): The Science and Practice of Clinical Medicine. New York, Grune and Stratton, Inc. In press.

8. Hosea, S.W. and Frank, M.M.: Hypocomplementemia. In Cohen, A.S. (Ed.): The Science and Practice of Clinical Medicine. New York, Grune and Stratton, Inc. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00046-10 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Pathogenesis of Delayed Hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.H. Kirkpatrick	Head, Clinical Allergy and Hypersensitivity Section	LCI	NIAID
Other:	A.I. Agbata	Visiting Fellow	LCI	NIAID
	M. Abedin	Guest Worker	LCI	NIAID
	J.I. Gallin	Senior Investigator	LCI	NIAID
	L.E. Greenberg	Biol. Lab. Tech. (Micro.)	LCI	NIAID

COOPERATING UNITS (if any) J. Cook and A. Lewis (LVD, NIAID, NIH); K. Hertz and S. Katz (Derm. Br., NCI, NIH) J. Twomey (Dept. of Medicine, Baylor Univ.) P.G. Sohnle (Dept. of Medicine, Medical College of Wisconsin).

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NIAID, NIH, Bethesda, Maryland 20014

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3 9/12	2 9/12	1

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SUMMARY OF WORK (200 words or less - underline keywords)

The nature of the immunologic defect in chronic mucocutaneous candidiasis has been further defined. Measurements of plasma thymopoietin-like activity yielded essentially normal results and indicated that the lesion was not due to inadequate inductive activity from the thymus. The measurements of LIF production by antigen-stimulated cells suggested that this lymphokine was not essential for development of delayed cutaneous hypersensitivity responses. Even though lymphocytes from many patients could not produce LIF in response to antigens, in most instances mitogenic stimulation did induce LIF production. This indicates that the cells are genetically capable of producing the lymphokine and suggests that the fundamental defect is either in generation of antigen-responsive cells or failure of antigen recognition at the cell membrane.

The studies of immunologic reconstitution and resistance to infections continue. The data indicates that both "candida-positive" and "candida-negative" transfer factor has efficacy but that long lasting benefits are more likely when candida-sensitive transfer factor is used.

Project Description:

Objectives (sub-project A):

1) Characterization of cellular immune responses in immunologically deficient patients with chronic infectious diseases;

2) Studies of the pathogenesis of acquired and congenital immunologic deficiencies;

3) Studies of lymphokine production by cells from normal and immunodeficient subjects;

4) Studies of developmental immunology including studies of the immunologic activities of human cord-blood lymphocytes and maturation of cell-mediated immune systems in Syrian hamsters.

Methods Employed:

The major portion of our studies of cellular immune deficiency have been conducted in patients with chronic mucocutaneous candidiasis. Approximately 50 patients with this syndrome have been evaluated. Evidence of immunologic deficiency has been found in 66% of the patients.

The general model for the pathogenesis of cellular immunodeficiency that we presented in 1971 still serves as a valid outline in which to conduct studies of host-defenses. According to this model patients with candidiasis developing during infancy or early childhood have a congenital basis for their immunologic deficits. Recently we have seen candidiasis patients who did not have clinical evidence of immunodeficiency during childhood or early adult life. Instead, they developed classical mucocutaneous candidiasis during the fifth or sixth decade. These patients have been used to study mechanisms of acquired cellular immuno-deficiency. Studies that have been completed to date involve measurements of plasma thymic hormone-like activity, antigen and mitogen-induced lymphocyte transformation responses and lymphokine production, enumeration of T-cells and B-cells and assessment of delayed cutaneous hypersensitivity.

In our 1971 model we proposed that lymphocytes may respond to antigens by replicating (lymphocyte transformation), by producing lymphokines or by developing cytotoxic activity. The first two responses have received intensive evaluation in our laboratory and data have been generated, both by ourselves and by others, that suggest that these responses may involve separate subpopulations of T-cells. The studies have been extended to measurements of another lymphokine, leukocyte migration inhibition factor (LIF), by antigen and mitogen-stimulated lymphocytes from patients with cellular immuno-deficiency syndromes and normal controls.

In addition, Dr. Agbata has been studying the phenomenon of spontaneous interaction between ovine erythrocytes and T-cells from normal and immuno-

deficient patients. He has shown that mitogen-stimulated lymphocytes, and under appropriate conditions antigen-stimulated lymphocytes, produce a soluble factor that enhances formation of rosettes by T-cells. He is now studying T-cell subpopulations and attempting to characterize the factor.

We have also investigated the mechanisms of inflammation in the skin of patients with chronic candidiasis. This study was undertaken in an effort to define the inflammatory pathways that were operative in patients who had intense cutaneous inflammation, but deficient cellular immunity. The experiments indicated that the complement system was being activated directly via the alternative pathway, probably by the polysaccharide components of the organism and this in turn generated inflammatory mediators.

The models of cutaneous candidiasis in guinea pigs have been extended. Basophils have been identified along the basement membrane zone in infected tissues and the relationship of epidermal proliferation to clearing of cutaneous candidiasis has been studied.

Two projects are directed toward elucidation of the immunologic functions of lymphoid cells from human neonates and newborn hamsters. These include elucidation of suppressor activities, expression of antigen and mitogen-stimulated responses and the roles of macrophages and soluble factors. Resistance to the oncogenic effects of adenovirus-transformed cells is being assessed in hamsters.

Major Findings:

The studies of the nature of the immunologic defects in candidiasis patients have been extended. Dr. Stanley Chapman established an assay for the lymphokine, leukocyte migration inhibitory factory (LIF) and has studied LIF production by antigen and mitogen-stimulated lymphocytes from candidiasis patients. These studies have shown that cells from most candidiasis patients respond to mitogens by releasing LIF, but responses to stimulation with antigens are variable. In general, cells from patients with active candidiasis failed to respond to *in vitro* stimulation with candida with LIF production, but once the patients are in remission, candida-induced LIF production was observed. These findings indicated that the patients' cells were genetically capable of producing LIF, but did not do so when the disease is active. This finding suggests that an active, suppressive process may be present. Other experiments have shown that serum factors are not responsible for this effect. An unexpected finding was the disparity between delayed cutaneous responses to candida and LIF production by candida-stimulated lymphocytes. Many candida-reactive patients failed to produce LIF, and this phenomenon indicates that this mediator is not essential for development of delayed hypersensitivity reactions in the skin.

The studies of rosette formation by T-cells have yielded evidence for a new lymphokine E-RAF). With the exception of a patient with SCID, both normal donors and immunodeficient patients have lymphocytes that respond to stimula tion with mitogens by producing E-rosette Augmenting Factor (E-RAF). In

contrast, production of this factor by antigen-stimulated cells correlates closely with the patients delayed cutaneous responses to that antigen. Current evidence indicates that this factor enhances the numbers of Tμ cells. It has not yet been determined if it functions by simply facilitating expression of latent Tμ cells or causes maturation of null cells.

The plasma thymopoietin-like activity in the serum of patients with candidiasis has been assayed by measuring appearance of Thy 1.2 on spleen cells of germ-free, nude mice. Two patients with thymus aplasia had no detectable thymopoietin-like activity in their serum, but developed this activity after receiving thymus transplants. Other candidiasis patients including patients with chronic diffuse candidiasis, candidiasis with endocrinopathy and candidiasis with thymoma had normal or elevated levels of serum thymopoietin-like activity.

The guinea pig model for cutaneous candidiasis has yielded an interesting finding. The cutaneous infections evoke an intense epidermal proliferation that causes sloughing of organisms. This reaction correlates with the presence or absence of delayed cutaneous hypersensitivity responses by the animal. Thus, it appears that epidermal proliferation is an expression of cell mediated immunity that provides a means for shedding superficial infections.

Human lymphocytes may develop suppressor activity when they are activated with concanavalin-A. In contrast, human cord blood-lymphocytes are suppressive of responses by adult cells even without concanavalin activation. Fetal cells appear to be in a "suppressive state" under normal conditions.

The studies of tumor immunity in the hamster have been rewarding. First, Dr. Cook characterized the ontogeny of development of Con A-responsive lymphocytes in the hamster. These experiments have shown that newborn hamsters have few Con A-reactive cells, but after the first week of life there are essentially no Con A responses. This condition persists until 24-27 days of life when there is a rapid increase in Con A-reactivity to the levels seen in adult animals. This event does not correlate with the development of resistance to oncogenesis by adenovirus transformed cells, an event that is essentially complete at 11 or 12 days of age. We have found that addition of syngeneic macrophages from adult hamsters to cell lymphocytes from younger (ages 9-17 days) hamsters make the lumphocytes responsive to concanavalin. Thus the developmental delay is due to macrophages, not lymphocytes.

Significance:

1) These experiments and those presented in sub-project B (below) further define the relationship between cellular immune responses, inflammation and susceptibility to certain chronic infectious diseases. The relative contribution of individual lymphokines to cell mediated immune responses is unclear. For example, it is not known which or how many lymphokines are

required to generate a positive delayed skin test. The experiments of Dr. Chapman show that LIF is probably not required for production of reactive skin tests.

Dr. Agbata's soluble factor has many properties of a lymphokine, and if so, it represents a heretofore unrecognized factor. It is produced by antigen-stimulated cells from skin test positive persons, but not be skin test-negative persons. It was not produced by cells from a patient with SCID. Another important part of his discovery is the simplicity of the assay. Measurement of MIF, LIF, and chemotactic lymphokines are difficult and avoided by many laboratories. The phenomenon of rosette formation is easy to measure and quantitate.

The work in guinea pigs has shown a relationship between delayed hypersensitivity and epidermal proliferation in response to epicutaneous antigens. This observation has potential application to the whole area of scaling dermatoses.

Sequential maturation of the functions of lymphoid cell populations occur during fetal life. Our studies have shown that in the hamster macrophage maturation lags behind maturation of the lymphocyte and that this event may be related to acquisition of resistance to certain tumors. The relationship of suppressor cells in human cold blood to the maternal-fetal tolerance is currently unclear.

Proposed Course:

Future efforts will be directed toward definition of the roles of various lymphokines in the pathogenesis of cellular immunity and anergy. Isolation and characterization of the factor that enhances rosette formation is planned.

Objectives (sub-project B):

1) Evaluation of immunologic reconstitution as a therapeutic adjunct for treating chronic infectious diseases;

2) Identification and characterization of the components of dialyzable transfer factor and assessment of their functions in immunologic and inflamatory reactions;

3) Investigation of transfer factor-like materials in laboratory animals.

Methods Employed:

The trial of transfer factor in candidiasis is being continued with the oldest participants being in the seventh year of treatment. These subjects receive transfer factor every four months. A companion study in which other immunodeficient candidiasis patients are being treated with transfer factor

from candida skin test negative donors.

Whole dialyzable transfer factor is being separated into its component parts by gel filtration, ion-exchange chromatography and silica gel chromatography. Fractions are assayed for composition and activities on immune responses in vivo and in vitro.

Major Findings:

Thus far seven patients in whom remissions were induced with amphotericin are receiving transfer factor from candida-sensitive donors. In each case the patient has developed in vivo and in vitro evidence of cellular immunity to candida. This indicates that the transfer factor was active and that transfer factor was capable of "correcting" the immunologic lesion in the patients' cells. Four of the patients have maintained positive skin responses to candida and all of these patients have remained in remission for 2 to 7 years. These remissions are substantially longer than those expected with amphotericin alone. The three patients who failed to maintain cellular immunity to candida also suffered relapses of cutaneous candidiasis.

Five patients have received transfer factor from donors who do not have cellular immunity to candida. All patients were first treated with amphotericin to induce remissions. None of the recipients have developed cellular immunity to candida, an observation that suggests that transfer factor has immunologic specificity. Two of these patients have relapsed. The other three are in remission and have been for periods of 8 months to 3 years.

In summary, all recipients of transfer factor (from either candida-sensitive or candida-insensitive donors) have remissions that are longer than those seen with amphotericin B alone. It is important to note that the relapses have occurred only in persons who did not achieve or maintain cell-mediated immunity to candida.

Significance:

These experiments have shown that transfer factor may have non-specific effects that provide some benefits to patients with chronic mucocutantous candidiasis. However, long term remissions only occur in recipients who acquire and maintain cellular immunity to candida.

Proposed Course:

The patients will be followed until each person has been under treatment for at least one more year. In addition, we will continue to pursue the chemical nature and mechanism of action of transfer factor and evaluation of a mouse model.

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2. Kirkpatrick, C.H. and Smith, T.K.: Specific and Non-specific Activities in Dialyzable Transfer Factor. In Lucas, D.O. (Ed.): Regulatory Mechanisms in Lymphocyte Activation. New York, Academic Press, 1977, pp. 174-188.

3. Wright, D.G., Kirkpatrick, C.H., and Gallin, J.I.: Effects of levamisole on normal and abnormal leukocyte locomotion. J. Clin. Invest. 59: 941-950, 1977.

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5. Lewis, V., Twomey, J.J., Goldstein, G.G., O'Reilly, R., Smithwick, E., Pahwa, R., Pahwa, S., Good, R.A., Schulte-Wisserman, H., Horowitz, S., Hong, R., Jones, J., Sieber, O., Kirkpatrick, C.H., Polmar, S., and Bealmear, P.: Circulating thymic hormone activity in congenital immunodeficiency. Lancet 2: 471-475, 1977.

6. Sohnle, P.G. and Kirkpatrick, C.H.: Epidermal proliferation in the defense against experimental cutaneous candidiasis. J. Invest. Dermatol. 70: 130-133, 1978.

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9. Gallin, J.I., Malech, H.L., Wright, D.G., Whisnant, J.K. and Kirkpatrick, C.H.: Recurrent severe infections in a child with abnormal leukocyte function: Possible relationship to increased microtubule assembly. Blood 51: 919-933, 1978.

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12. Kirkpatrick, C.H.: Transfer of delayed cutaneous hypersensitivity with transfer factor. Cell. Immunol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00047-09 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Clinical studies on patients with known or suspected Parasitic diseases.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PIs:	F. A. Neva	Head, Clinical Parasitology Section, LCI, NIAID (dual position)
	T. E. Nash	Senior Investigator, Clinical Parasitology Section, (dual position), LCI, NIAID
	E. A. Ottesen	Senior Investigator, Clinical Parasitology Section, (dual position), LCI, NIAID
	D. J. Wyler	Senior Investigator, Clinical Parasitology Section, (dual position), LCI, NIAID
Others:	R. D'A. Gusmão	Visiting Fellow, LPD, NIAID
	T. Lawley	Medical Investigator, NCI
	H. Jane Blackman	Senior Investigator, NEI
	M. N. Lunde	Staff Member, LPD, NIAID
	J. Chulay	Medical Officer, WRAIR

COOPERATING UNITS (if any)
San Juan Laboratory, CDC; Tuberculosis Chemotherapy Center, Madras, India (Dr. S. P. Tripathy); Medical College of Madras, India (Prof. K. V. Thiruvengadam); National Eye Institute; Walter Reed Army Institute of Research;

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Parasitology Section

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NIAID, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The pathogenesis of chronic Chagas' disease could not be related to lymphocyte blastogenic responsiveness to antigens of the causative trypanosome. Some suggestive associations with absence and presence of certain HLA tissue types, however, were found in chronic Chagas' patients. Although specific cell mediated immune responses to parasite antigens in patients with leishmaniasis were demonstrated they must be differentiated from non-specific T-cell stimulation which can occur. Possible auto-immune responses to retinal antigens is being studied in patients with toxoplasmic chorioretinitis.

A high degree of specific IgE response as measured by histamine release from white cells challenged with microfilarial antigens was demonstrated in patients with tropical pulmonary eosinophilia. In contrast, histamine release of white cells from filarial patients with microfilaremia was low. A similar lack of immune responsiveness by lymphocyte transformation in microfilaremic patients seems related to both serum and cellular inhibitory factors.

Circulating immune complexes that bind complement were demonstrated in patients with acute schistosomiasis. These complexes decreased after treatment

Cooperating Units, cont'd:

Federal University of Goias, Goiania, Brazil (Profs. J. Rezende and A. Rassi); Federal University of Brazilia (Prof. A. Prata); and Duke University Medical Center (Dr. F. Ward). See also Project Reports Nos: 00102-04, 00161-02, 00093-03 and 00141-03 for LPD, NIAID.

Summary of work, cont'd:

or as the infection became chronic. Antibody response in the IgM fraction to a specific schistosome antigen was found to be characteristic of early infections in man.

Project Description:

This report covers only the clinically related research carried out by the four investigators in the Clinical Parasitology Section (Drs. Neva, Nash, Ottesen and Wyler). These senior investigators continue to provide a consultation service in clinical parasitology for the Clinical Center as well as participating with other LCI Staff in clinical infectious disease coverage at the Clinical Center and Naval Medical Center. Research not involving patients carried out by members of the Clinical Parasitology Section is reported in the Annual Report from LPD. Over the past year much of the clinical investigation was done abroad in India and Brazil and with collaborating groups from the Eye Institute and Walter Reed Army Institute of Research.

Immune response to intra-cellular protozoan infections.

Several aspects of immune response to intra-cellular protozoan infections are being studied. In the case of cutaneous leishmanial infections it has been demonstrated that specific stimulation of T cells usually occurs but some patients with early infections lack this response. However, under certain conditions non-specific T cell stimulation can also occur. But the immunologic events responsible for killing of leishmania remain unknown. (Wyler and Chulay). Immune responses to another intracellular parasite, Toxoplasma gondii, are being studied in an effort to understand the pathogenesis of toxoplasmic chorioretinitis. Preliminary studies indicate that some patients with this condition have lymphocytes in their circulation that have been sensitized with retinal antigens. One patient was found to have circulating antibody to retinal antigens. The significance of these findings in terms of an auto-immune mechanism for toxoplasmic chorioretinitis will be evaluated (Wyler and Blackman).

Since clinical manifestations of chronic Chagas' disease occur after a latent period of many years in relatively few of those individuals who have been infected by the intracellular parasite, Trypanosoma cruzi, clues were sought for possible immunologic participation in the pathogenesis of this disorder. In collaboration with Brazilian colleagues lymphocyte blastogenesis tests in carefully selected patients with the cardiac and esophogeal forms of chronic Chagas' disease, as well as in appropriate controls, were carried out using various antigen preparations of the causative parasite, T. cruzi. Most of the antigens tested were capable of eliciting blastogenic reactions, but blastogenic responses varied widely; moreover, the results in patients with obvious chronic Chagas' disease could not be differentiated from those

individuals who have been infected by the parasite but have no evidence of disease . (Neva, Gusmao and Brazilian collaborators). Another part of the Brazilian study involved determination of HLA types at the A and B locus in patients with Chagas' disease. One HLA type was found to be underrepresented and two antigens in somewhat higher frequency than in Brazilian control groups, but the statistical significance of these associations is still borderline. Further tissue typing studies are planned. (Neva, Gusmão, Duke University and Brazilian collaborators).

Immune response to helminthic infections.

Both IgE-mediated allergic reactions as well as cell-mediated lymphocyte blastogenic activity were studied in patients with filariasis in India. A striking degree of immunologically specific allergic response as measured by histamine release from white cells exposed to microfilarial antigens was found in those patients with the form of disease called tropical pulmonary eosinophilia, characterized by asthmatic symptoms, marked eosinophilia but no circulating microfilariae. Paradoxically, white cells from those patients with microfilaremia failed to release histamine when exposed to the same antigens. A similar lack of immune responsiveness as measured by lymphocyte transformation to filarial antigens is found in some patients with filariasis. In this case immune unresponsiveness appears to be caused by suppressor cells and inhibitory serum factors. (Ottesen and Indian collaborators).

Inhibitory serum factors and an adherent fraction of suppressor cells were also found in patients with chronic schistosome infections. The immunologic unresponsiveness was antigen specific. Patients with acute schistosomiasis, who incidentally have brisk blastogenic responses to schistosomal antigens, were found to have Cl_q binding immune complexes in their circulation which declined after treatment or with time as the infection became chronic. (Ottesen and Lawley).

The magnitude of post-treatment eosinophilia in patients with Bancroftian filariasis was found directly related to numbers of microfilariae in the circulation. (Ottesen).

The class of immunoglobulin responding to the polysaccharide gut antigen of schistosomes was found to be useful in assessment of duration of human schistosome infections. Specific anti-gut antibody was highest in the IgM fraction of patients with recent infections. (Nash and Ottesen).

Publications:

Neva, F.A.: Looking back for a view of the future: Observations on immunity to induced malaria. Am. J. Trop. Med. Hyg. 26: 211-215, 1977.

Nash, T.E., Masir-ud-Din and Jeanloz, R.W.: Further purification and characterization of a circulating antigen in schistosomiasis. J. Immunol. 119: 1627, 1977.

Weintraub, M., Nash, T.E., Ottesen, E.A., and Becker, M.A.: Uricosuril effects of Niridazole. Clin. Pharmacol. Thera. 22: 568, 1977.

Ottesen, E.A., Stanley, A.M., Gelfand, J.A., Gadek, J.E., Frank, M.M., Nash, T.E., and Cheever, A.W.: Immunoglobulin and complement receptors on human eosinophil and their role in cellular adherence to schistosomules. Am. J. Trop. Med. Hyg. 26: 134, 1977.

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Neva, F.A., and Ottesen, E.A.: Tropical (Filarial) Eosinophilia. New Eng. J. Med.: 298: 1129-1131, 1978.

Ottesen, E.A. and Cohen, S.G.: The Eosinophil, Eosinophilia, and Eosinophil-Related Disorders. In Middleton, E., Ellis, E. and Reed, C.E. (eds.): Allergy: Principles and Practice. C.V. Mosby Co., St. Louis, Mo. pp 584-632, 1978.

Weller, P.F., and Ottesen, E.A.: Failure of Diethylcarbamazine as a Provacative Test in Subperiodic Wuchereria bancrofti Filariasis. Trans. Roy. Soc. Trop. Med. Hyg. 71: 31-32, 1978.

Miller, L.H., Neva, F.A., and Gill, F.: Failure of chloroquine in human babesiosis (Babesia microfti): Case report and chemotherapeutic trials in hamsters. Ann. Internal. Med. 88: 200-202, 1978.

Wyler, D.J.: Leishmaniasis in Current Therapy, 1979 (H.F. Conn, ed).

Nash, T.E.: Antibody response to a polysaccharide antigen in schistosomiasis. I. Sensitivity and specificity. Am. J. Trop. Med. Hyg. (In press).

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Ottesen, E.A., Hiatt, R.A., Cheever, A.W., Sotomayor, and Neva, F.A.: The Acquisition and Loss of Antigen-Specific Cellular Immune Responsiveness in Acute and Chronic Schistosomiasis in Man. Clin. Exp. Immunol. (In press).

Ottesen, E.A.: Filarial Infection and the Host Response in Man. Paradoxes and Insights. in Escape from Immune Surveillance: The Interface Between Immune Mechanisms and Disease. D.B. Amos, R.S. Schwartz and B.W. Janicki, eds. Academic Press (In press).

Ottesen, E.A.: Visceral Larva Migrans and Other Unusual Helminth Infections of Man. in Principles and Practice of Infectious Disease, Mandell, G.L., Douglas, R.G., and Bennett, J.E. eds. J. Wiley and Sons, New York. (In press).

Lunde, M.N., Ottesen, E.A., and Cheever, A.W.: Serological Differences Between Acute and Chronic Schistosomiasis mansoni Detected by Enzyme Linked Immunosorbant Assay (ELISHA). Am. J. Trop. Med. Hyg. (In press).

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Wyler, D.J., Oster, C.N., and Quinn, T.C.: The role of the spleen in malaria infections. Bull. W.H.O. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00048-08 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Pathophysiology of Autoimmune Hemolytic Anemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immunology Section and Chief, LCI	LCI	NIAID
OTHER:	M. Hamburger	Clinical Associate	LCI	NIAID

COOPERATING UNITS (if any) T. Lawley (Dermatology Branch, NCI, NIH); P. Plotz (Arthritis Branch, NIAMDD, NIH); H. Moutsopoulos, and T. Chused (NIDR)

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SECTION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have developed new assays for the functioning of IgG Fc receptors and complement receptors in various groups of patients with immunologic disease. All patients with active lupus erythematosus have major abnormalities in their Fc receptors. Patients with mixed IgM-IgG cryoglobulins, although they have high levels of circulating immune complexes, have no such abnormalities. These patients of course do not usually develop renal disease. Patients with Sjogren's syndrome have been divided into two groups, those with Fc clearance defects, and those with normal Fc specific clearance. The patients with normal Fc receptor function have disease which is confined to the glands of the face and head. Patients with abnormal Fc receptor function have wide-spread illness which includes rheumatoid arthritis, interstitial nephritis, interstitial pulmonary lung disease, and other signs of extra facial immunologic disorders. The results suggest that this important assay can divide patients with various kinds of immunologic abnormalities into pathophysiologic groups.

Project Description:

Objectives:

To define the pathophysiologic function of antibody and complement on the surface of red cells in patients with hemolytic anemia, and to use the findings which have been developed from this study to develop new tests for immunologically specific receptors function in a variety of disease states. The tests developed for the study of autoimmune hemolytic anemia have been found to have wide application to other autoimmune and immunologic problems.

Methods Employed:

These studies examine the clearance of radiochromated erythroctyes from the circulation. The initial studies were performed in a guinea pig model, but almost all of the recent studies have been performed in human volunteers or in patients with various diseases. In the initial studies in humans, the clearance of erythrocytes coated with IgM isoagglutinin antibodies was examined. In later studies radiochromated erythrocytes were coated with highly purified human IgG anti-Rh antibodies and the survival of these cells was also studied. The third general set of clearance studies examined the clearance of radioactive albumin which had been aggregated. This is the standard method for determining RES clearance and can be used to determine both liver blood flow and the activity of the reticuloendothelial system.

Major Findings:

In the past years, we identified the immunologically active protein fragments which, when deposited on red cells, are responsible for the clearance of those cells from the circulation. It was reported that receptors for these immunologically specific fragments were present on the membrane of macrophages which were responsible for removal of cells coated with these fragments from the circulation. It became clear that it was possible to study patients with a wide variety of diseases to determine whether their receptors for these immunologically active fragments were functioning normally in vivo. In the past it has been known that the reticuloendothelial system (RES) plays a major role in removing foreign material from the circulation. These foreign materials include bacteria and parasites, as well as endogenously generated immunoreactive materials such as immune complexes. It was suspected that defects in RES function might be important in both infectious and immunologic diseases. However, when this question was examined directly using the techniques available, no defects in RES function were noted in any disease state in which blood flow to the RES organs was normal. Until the present studies, the techniques available for examination of RES function was that of study of clearance of aggregated albumin from the circulation. This study has led to the development of new techniques for evaluation of RES function. It has become clear that there are indeed diseases with defects in RES function if one examines this question looking

at specific receptors for immunologically active materials. Thus receptors for complement and immunoglobulin on a patient's macrophages will recognize these immunologically active materials coating foreign substances as they circulate. Our test utilizes the patient's own red blood cells. The cells are coated with antibody and/or complement fragments and are infused into the patient and their clearance is followed.

Using this technique we have found that every patient we have studied with active lupus erythematosus has a major defect in Fc receptor specific clearance although the clearance of aggregated materials is normal. We believe that it is quite likely that this clearance defect applies to the clearance of circulating immune complexes which occur in these patients. If such complexes were not cleared efficiently from the circulation, they could be deposited in the glomeruli where they could cause renal disease. We have also studied patients with lupus serially over a period of time. We find that as the disease improves, the clearance defect also tends to improve and the complexes present in the ciruclation tend to disappear. However it is striking that some patients with lupus erythematosus have a persistance of their clearance defect for long periods of time, even on treatment. The next major area of study has involved examination of patients with Sjogren's syndrome. With Drs. Moutsopoulos and Chused we have had an opportunity to examine a very large number of patients with this syndrome. Using the Clq binding assay, established by Dr. Lawley in our laboratory, we find that 86% of the patients with Sjogren's syndrome have evidence of ciculating Clq-binding complexes. Nevertheless, the patients clearly divide into two groups with regard to Fc receptor specific clearance. There are some patients with a defect in clearance and other patients who do not have a defect in clearance. When the data was critically evaluated, it was striking that the patients with no clearance defect had disease which was confined to the glands of the face and head. They did not have the extra-facial manifestations of Sjogren's syndrome. On the other hand, examination of patients with clearance defects showed that almost all of these patients had disease which was spread beyond the face and head. Thus these patients had interstitial nephritis, interstitial pulmonary disease, rheumatoid arthritis, etc., all extra-facial manifestations of this devastating illness. Once again we believe that our tests of Fc receptor function are showing us fundamental differences in the status of patients with this disease. There may be differences in the complexes that circulate in these two patient groups and more importantly the test may act as a prognostic indicator. Two of the patients that we had an opportunity to study with disease confined to the face and head had an abnormality in clearance function. Both of these patients went on to develop extra facial manifestations of their disease. The numbers are still too small to know whether this difference will be absolute, hoever, the findings are quite striking at present.

Proposed Course:

At the present time we are extending these studies to patients with mixed connective tissue diseases as well as patients with mixed cryoglobulinemias. We already know that the patients with mixed connective tissue disease have no clearance defect, and there is, in general, no defect in

the patients with the mixed cryoglobulins. This group of patients will be expanded considerably. We believe that these studies can be applied to a number of autoimmune diseases and a number of infectious diseases. We hope that they will directly lead to an evaluation of the immune complexes present in these diseases and that we will be able to correlate the nature of the circulating complex with whether or not the patient has a receptor defect. This argument is predicted on the belief that the receptor defect is due to competitive inhibition of receptor function by circulating complexes. It is of course possible that this disease is not due to inhibition of receptors but due to a more fundamental defect in the generation of receptors that allows the circulating complexes to continue to circulate. If this were the case, such a defect might predispose individuals to the development of diseases such as lupus erythematosus and its complications. We have no evidence to suppost this contention at the present time, but it is an exciting prospect and would give some great insights into the understanding of lupus if it were indeed true. Serial studies of patients with these various diseases should allow us to begin to come to grip with this question in the future.

Publications:

1. Frank, M.M., Schreiber, A.D., Atkinson, J.P., and Jaffe, C.J.: Pathophysiology of immune hemolytic anemia. Ann. Intern. Med. 87:210-222, 1977.

2. Lawley, T., and Frank, M.M.: Antigen-Antibody Complexes and Immune Complex Diseases. In Parker, C. (Ed.): Clinical Immunology. Philadelphia, PA, W. B. Saunders Co. In Press.

3. Jones, E.A., Frank, M.M., Jaffe, C.J., and Vierling, J.M.: Primary Biliary Cirrhosis and the Complement System. Ann. Int. Med. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00049-08 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Initiation and Regulation of Antigen Recognition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	J. T. Blake	Bio. Lab. Tech. (Micro.)	LCI	NIAID
	K.U. Cehrs	Bio. Lab. Tech. (Micro.)	LCI	NIAID
	K. Yokomuro	Guest Worker	LCI	NIAID
	J. Schroer	Guest Worker	LCI	NIAID
	J. Thomas	Clinical Associate	LCI	NIAID
	R. Clark	Clinical Associate	LCI	NIAID

COOPERATING UNITS (if any)
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SECTION
Biologic Structure Section

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interests and proposed course of the Biologic Structure Section is to characterize the role of the macrophage in antigen recognition by T lymphocytes in the expression and initiation of cell-mediated immunity in man and experimental animals. More specifically, we are interested in exploring the role of the major histocompatibility complex in the genetic regulation of cell-cell interactions required for expression of cell mediated and humoral immunity. The Biologic Structure Section is also actively characterizing the physical state, localization, and fate of macrophage associated antigen. Additional studies of genetics of immune responsiveness to insulin done in man will be assessed and compared to mouse and guinea pig. Determinant selection by macrophages functionally describes Ir gene control of antigen recognition by T lymphocytes in experimental animals. An understanding of the operation of a similar mechanism in man may have implications in the pathophysiology of insulin allergy in diabetics.

Project Description:

Objectives:

1) To characterize in a quantitative and qualitative fashion the role of macrophages and lymphocytes in a variety of in vitro correlates of cellular and humoral immunity such as antigen-dependent lymphocyte proliferation and generation of antigen specific plaque formation.

2) To study in experimental animals the mechanism of lymphocyte antigen recognition using defined antigens such as insulin.

3) Characterize the role of macrophage-lymphocyte interaction in regulation of antigen recognition by thymus-derived and bone-marrow derived lymphocytes.

4) To study the cellular and molecular basis of the genetic control of immune responsiveness and the role of surface membrane determinants in cell cooperation.

Methods Employed:

1) Determination of DNA synthesis of mouse and guinea pig lymphocyte populations with and without addition of antigen by 4 hour pulse of ^{3}H-TdR incorporation. Results assayed by either/or both autoradiography or liquid scintillation spectrometry.

2) Preparation and isolation of partially purified population of lymphocytes from oil-induced peritoneal exudates or regional draining nodes subsequent (2-4 weeks) to immunization of inbred strain 2 and 13 guinea pigs with chemically defined antigens such as horse spleen ferritin, horseradish peroxidase or hapten protein conjugates.

3) Column purification: Peritoneal exudate cells (PEC) or lymph node cell suspensions were passed over glass bead columns and incubated at 37°C for one hour. Cells were then eluted with 10% guinea pig sera in minimal essential Eagle's media and the recovered cells studied.

4) Preparation of macrophage-rich oil-induced peritoneal exudate cells.

5) Binding of lymphocytes to a macrophage receptor can be assessed both in the presence and absence of antigen. Monolayers of guinea pig macrophages are prepared by adherence of monocyte-macrophages from oil-induced peritoneal exudates to Lab-tek tissue culture chamber slides. After removal of non-adherent cells $7x10^{6}$ lymphocytes are added to either control or antigen pulsed monolayers for varying periods of time, after which the non-macrophage bound lymphocytes are removed by washing and the monolayer fixed with 1% gluteraldehyde for light or electron microscopic analysis.

6) The uptake and fate in culture of soluble protein antigens is being studied functionally by metabolism and cellular localization.

7) Assay of the mixed leukocyte reaction (MLR) by assay of ^{3}H-TdR incorporation occurring when cells from a variety of strain 2 and 13 guinea pig lymphoid tissues are mixed and cultured for 5-7 days in vitro.

8) Carrier and hapten specific plaque forming cell assays by the assay Jerne.

Major Findings:

A. Background Information

It is generally recognized that macrophages facilitate a variety of in vitro thymic-dependent immunological phenomena in the mouse, guinea pig, and man. We have analyzed the cellular and molecular events which underlie their function. Antigen specific activation of mediator production and clonal expansion of T lymphocytes requires the presence of macrophages, and the effects of macrophages in such activation extend beyond maintenance of in vitro culture conditions. Indeed in the guinea pig, one can clearly demonstrate that recognition of soluble protein antigens by the T lymphocyte requires an initial uptake of antigen by the macrophages.

By contrast the role of macrophages in stimulation of B lymphocytes by specific antigen differs significantly from that played for T cell activation. Macrophages suppress proliferation of B cell populations depleted of T lymphocytes. Indeed proliferation of B cells appears to be more specifically under the influence of soluble factors elaborated by T cells subsequent to antigen-specific activation.

The biologic constraints inherent in a two cell immunogenic recognition unit are clear. It is necessary to define both the cellular and immunological specificity of this requirement for macrophages.

The role of macrophages in antigen specific proliferation in the mouse has been examined. Our data indicate that unlike the guinea pig macrophage-dependent support of the proliferation of lymph node lymphocytes is not restricted to syngeneic macrophages. Efforts to resolve this apparent paradox are currently under investigation.

Is macrophage lymphocyte interaction a general requirement for all T cell activation or do these associations function solely in the recognition of cell bound and soluble protein antigens? In work done by our section the response of T cells to PHA was shown to also require accessory cells; but unlike the macrophage requirement for soluble protein antigen recognition, their function can be replaced by fibroblasts or increased cell density.

Macrophage lymphocyte interaction may occur either indirectly via the secretion of antigen complexed to soluble macrophage factors cytophilic for lymphocytes or via direct cell interaction. Our section has shown at least two types of physical interactions to take place. The first occurs rapidly, is independent of the presence of antigens, and is without immunologic

commitment. This step requires active macrophage, metabolism, divalent cations, and involves a trypsin sensitive macrophage site. This binding does not distinguish T or B cells and is reversible such that at any given time binding represents an equilibrium between cellular association and dissociation. When this step has brought specifically immune lymphocytes into apposition with antigen bearing macrophages, a second type of binding results. This latter phenomenon is dependent upon the presence of antigen and a sharing on lymphocyte and macrophage of histocompatibility linked gene products. This association is not easily reversed and eventuates in proliferation of the bound lymphocyte.

The use of antigens under control of dominant H-linked genes and specific alloantisera which recognize cell associated antigenic determinants which are also products of H-linked genes provide useful biological probes of the function and cellular location of molecules which play critical but incompletely defined roles in the T lymphocyte antigen selection or activation process. Alloantisera block the antigen response controlled by an IR gene linked to the gene coding for the alloantigen, but had little effect on the antigen response controlled by an Ir gene which is the product of the opposite haplotype. The inhibitory activity of the alloantisera was initially presumed to be directed solely against the proliferating T lymphocyte. However, the central role of the macrophage in the activation of T cell proliferation suggests that an alternative site for the inhibitory effect of the alloantisera might be on the macrophage itself by blockade of the physical and/or functional interaction of the macrophage and T cell. Unfortunately experiments which attempt to define the cellular site of action of the alloantisera using combinations of antigen pulsed macrophages and T cells are not possible because efficient interaction of macrophage associated antigen and T cell is only seen when the macrophage and T cell share identity of gene products linked to the guinea pig MHC.

There are, however, a number of special conditions in which a macrophage may "paradoxically" induce allogeneic lymphocyte proliferation. These circumstances are rather revealing situations, in that they have clearly shown that the failure to recognize soluble protein antigens bound to allogeneic macrophages is not a primary inability of such cells to present signals but may instead reflect a basic feature of antigen handling by macrophages.

Our lab has shown guinea pig macrophages to be very effective stimulators of allogeneic lymphocyte proliferation. Thus soluble proteins may be displayed in an immunologically relevant fashion only on syngeneic macrophages; whereas in the MLR, it is the histoincompatible macrophage that induces T cell proliferation, again suggesting that the critical difference lies in the display of alloantigen as opposed to that of foreign proteins.

Based on the results of studies pursued in our section, models for the role of the macrophage in genetic control of T cell responses to soluble protein antigens must consider: 1) an antigen specific receptor on the T cell, 2) cellular interaction structures identical to or closely linked to the alloantigens, 3) the IR gene product functioning either as the specific

T cell receptor or as an auxillary antigen recognition molecule on the macrophage and/or T cell, 4) antigen bound to metabolically intact macrophages in a form resistant to proteolytic treatment and located in a cellular site not available to specific antibody, and 5) a macrophage associated nonspecific membrane trigger site or secretory product as suggested by the activation of aldehyde treated lymphocytes by normal macrophages.

At present, our section favors the hypothesis which states that a given macrophage's repertoire of IR gene products function to specify determinant selection. Possible mechanisms by which the interaction of antigen with the IR gene product could select determinant specificity are as follows:

1) Enzymatic - The IR gene product is an enzyme of relative broad specificity such as a protease which destroys only those polypeptide sequence towards which it has reactivity.

2) Conformational - The IR gene product alters the tertiary structure of the antigen itself so as to display selected regions of the polypeptide to the T cell.

3) Receptor Presentation - This would require that certain similar existing amino acid sequences or conformations in a complex antigen be recognized by IR gene products. Such receptor molecules would possess specific binding properties akin to that of the binding site of a proteolytic enzyme but would differ from the enzymatic model in that no selective degradation of the antigen occurs. The function of such class specific antigen binding molecules would be to orient or display those regions of the polypeptide antigen not bound to the receptor.

B. Genetic Control of the Immune Response to Insulin in the Guinea Pig.

Genetic control of immune responsiveness to both synthetic and natural polypeptide antigens has been found in experimental animals. Unfortunately absolute demonstration of such phenomenon in man has not been possible because of the limited number of ethical antigens which can be used in man and their lack of defined physical structure. One antigen which might be of significant potential in such studies is insulin. Beginning with studies in inbred Strain 2 and 13, we have found that cellular and humoral immune responsiveness of distinct regions of the molecule are under control of histocompatibility-linked genes. Strain 2 guinea pig T cells (as measured by proliferation or T cell help) recognize a determinant consisting o amino acids 6-11 of the A chain (αloop) while Strain 13 guinea pig T cells recognize a region in the insulin B chain. Nonetheless, the determinants recognized by antibody generated in both strains predominantly recognize shared determinants.

C. Determinant Selection: A Macrophage Ir Gene Function.

Although immune specificity is traditionally considered a lymphocyte function, observations from the above as well as other studies suggest an

interpretation that the macrophage has some discriminatory function in the antigen recognition process.

We thus assessed the function of macrophages in Ir control taking advantage of the capacity of T cells from insulin immunized F_1 (2 X 13) guinea pigs to respond to antigen bearing parental macrophages. We reasoned as follows:

If Ir gene function operates exclusively at the level of the responding lymphocyte, both determinants should be simultaneously recognized by F_1 T cells independently of which parental macrophage is "presenting" the antigen. Conversely, if the definition of which determinant is actually recognized depends on the genetic profile of the antigen presenting macrophage, then IR gene function must also be operating at this cell level. The data favor the latter assumption. Indeed our studies of the immune response to insulin, raise the possibility that a selected amino acid sequence and/or conformation within the antigen itself is seen by the T cell receptor. Generation or display of such antigenic determinants would thus be a function of immune response genes operating at the level of the antigen presenting cell. Two general mechanisms by which macrophages might subserve such a function may be proposed. One possibility is that immune response genes define a class of receptors or broad specificity which recognize molecular shape and thus have the unique ability to focus or orient distinct regions of the antigen for presentation to the T cell. A second possibility is that immune response gene products are or regulate the activity of families of enzymes which modify or metabolize polypeptide antigens. In this latter situation, the repertoire of IR genes associated with a given haplotype would define restricted areas of the molecule as available for display to the T cell receptor. Experiments to distinguish these possibilities are in progress.

D. Role of Adherent Cells in Murine T Cell Antigen Recognition (Rosenwasser and Rosenthal)

The cellular requirements for murine T cell antigen recognition are less well understood and were therefore evaluated in an adherent cell dependent in vitro assay. The immune response of sensitized T cells to complex multideterminant soluble protein antigens such as dinitrophenylated ovalbumin is dependent on the production of a soluble factor which is not antigen specific nor H-2 related in that adherent cells, regardless of haplotype produce factor independent of the presence of antigen. Mouse lymphocytes do not, however, respond to supernates of either guinea pig macrophages nor fibroblast, indicating the existence of species and cell type specificity. By contrast the adherent cell requirement for T cell proliferation to the synthetic terpolymer L-glutamic acid, L-lysine, L-tyrosine (GLT^{15}), an antigen, the response to which is under specific Ir gene control, necessitated identity or partial identity at the major histocompatibility complex between macrophage and primed T cell.

E. Genetic Control of the Insulin Immune Response in Mice

When the immune response to insulin is examined in the mouse by assay of T cell proliferation and anti-insulin antibody, a pattern of responder and nonresponder strains emerged. The results are summarized below.

	Pork insulin	Beef insulin	Pork proinsulin C peptide
$H\text{-}2^b$	-	+	-
$H\text{-}2^d$	+	+	-
$H\text{-}2^k$	-	-	+
$H\text{-}2^q$	-	-	-

These results (Rosenwasser and Rosenthal) as well as studies using H-2 recombinants on a B^{10} background demonstrate that the immune response to insulin in the mouse is also controlled by MHC--linked Ir genes which map to the I-A subregion.

More recently Schroer in collaboration with John Inman of the Laboratory of Immunology has developed a method for coupling insulin to SRBC permitting a direct analysis of insulin specific antibody responses by plaque techniques. Examining the kinetics and compartmentalization of immune response in mice to insulin, Schroer and Thomas have found that IgG antibody formations begins in the node and appears in the spleen only after secondary immunization. Using these observations and in vivo cell transfers studies are now in progress to determine the T and B cell repretoire of specificity in positively selected virgin and insulin immune mice following alloagressive response to various MHC loci.

F. Determinant Specific Suppression of the Immune Response (Baskin and Rosenthal)

Prior (7-21 days) immunization of guinea pigs with multideterminant or defined protein antigens in incomplete adjuvant leads to specific unresponsiveness only to that antigen but not to an irrelevant antigen. This suppression was studied in the insulin model system using 2, 13 and F_1 (2 X 13) guinea pigs. As with help and proliferation following classic CFA plus insulin immunization, specific unresponsiveness is determinant specific. More importantly suppression can be transferred to normal animals by cells but not by serum. The precise character of the suppressor cell and its mechanism of action is under further study.

G. Macrophage Presentation of Antigen Determines MHC-linked Restrictions In Vivo Primary Immune Responses.

Significant controversy exists as to whether the observed MHC-linked restriction in antigen presentation in secondary in vitro and in vivo responses are the consequence of restrictions generated as a result of the

primary investigation or are pre-existing in the germ line. To answer the question Kozo Yokomuro has developed a method for in vivo priming of strain 2 and 13 guinea pigs with antigen bearing macrophages. His data show that antigen priming by macrophage does not occur across MHC barrier and that in F_1 guinea pigs mimicry is generated on for antigen bound to the original macrophage used for primary. Moreover when assessing immune responses by their approach using antigens under control of MHC linked Ir genes, on parental macrophages of responder origin elicit an immune response.

H. Immune Response Gene Control of the Immune Response to Insulin in Man.

In collaboration with Drs. Ron Kahn of the NIAMGD, Dean Mann of the NCI and John Galloway of the Lily Research Clinic we have examined the character of the immune response to insulin diabetics allergic to various therapeutic forms of insulin. After 2 years of preparation and study we have examined nearly 150 patients. Consonant with our studies in mice and guinea pigs there appears to exist a remarkable conservation in evolutionary tissue of discrete immune responses to restricted regions of the A and B chain. We have also analyzed both T and B cell responses and their correlation with HLA and B cell antigen typing. Results of these studies are in progress and will be completed before December 1978.

Significance to Biomedical Research and the Program of the Institute:

The characterization of the immune responsiveness of thymus-derived lymphocytes should provide a useful system for study of the role of the cellular immune effector cells in immunologic surveillance and their contribution to the humoral limb of host defenses. Tumor immunity and transplantation immunity have been shown to rely to a great extent upon mechanisms of cellular immune function such as cytotoxicity and elaboration of other so-called "soluble mediators". A more fundamental understanding of the immunobiology of these crucial events may provide new insights into immune competence in man and information on the mechanisms of cellular regulation of immune responsiveness. Potential approaches to therapeutic manipulation of such events would be of immediate clinical relevance. This is particularly evident from our studies of the immune response to insulin in man where precise determinations of etiology of the allergic response influences our therapy.

Proposed Course:

1) Characterize the nature of macrophage associated antigen.

2) Develop in the mouse assays for antibody formation using antigens of restricted heterogeneity such as insulin.

3) Establish in the guinea pig and mouse models of antigen specific suppression and explore the influence of MHC and non-MHC gene products on such phenomenon.

4) Assess further the immune response to insulin in man and define the presence or absence of Ir gene control and their association with the human MHC.

Publications:

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3. Barcinski, M.A. and Rosenthal, A.S.: Antigen mapping for T cell recognition: Immune response gene control of determinant selection. In Lucas, D.O. (Ed.): Proceedings of the Eleventh Leukocyte Culture Conference. pp. 382, 1977.

4. Ellner, J.J., Lipsky, P.E., and Rosenthal, A.S.: Antigen handling by guinea pig macrophages: Further evidence for the sequestration of relevant for activation of primed T-lymphocytes. J. Immunol. 118: 2053-2057, 1977.

5. Rocklin, R.E. and Rosenthal, A.S.: Evidence that human leukocyte inhibitory factor (LIF) is an esterase. J. Immunol. In press.

6. Rosenwasser, L.J. and Rosenthal, A.S.: Role of adherent cells in murine T cell antigen recognition. In Sercarz, E., Herzenberg, L. and Fox, C.F. (Eds.): The Immune System: Genes and Regulation (ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. VI). New York, Academic Press. pp. 429-437, 1977.

7. Scott, R.E. and Rosenthal, A.S.: Isolation of receptor bearing plasma membrane visicles from guinea pig macrophages. J. Immunol. 119: 143-148, 1977.

8. Rosenthal, A.S., Rosenwasser, L.J., and Barcinski, M.A.: Determinant selection: A macrophage-mediated immune response gene function. In McDevitt, H.O. (Ed.): Ir Genes and Ia Antigens. New York, Academic Press. 405-415, 1978.

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10. Rosenwasser, L.J. and Rosenthal, A.S.: Adherent cell function in murine T lymphocyte antigen recognition, II. Definition of genetically restricted and non-restricted macrophage functions in T cell proliferation. J. Immunol. (in press).

11. Rosenthal, A.S.: Determinant selection and macrophage function in genetic control of the immune response. Immunol. Rev. 40: 136-152, 1978.

12. Stingl, G., Katz, S.I., Shevach, E.M., Rosenthal, A.S., and Green, I.: Analogic functions of macrophages and langerhans cells in the initiation of the immune response. J. Invest. Dermat. (in press).

13. Feldmann, M., Rosenthal, A.S., and Erb, P.: Macrophage-lymphocyte interaction in immune induction. Int. Rev. Cytology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00050-08 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Clinical Studies of Complement Abnormalities of Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immunology Section and Chief	LCI NIAID
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	R. Reinhart	Bio. Lab. Tech. (Biochem.)	LCI NIAID
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Clinical Immunology

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NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 8/12	2 2/12	6/12

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hereditary angioedema is a disease inherited as an autosomal dominant trait. It is characterized by abnormality in a serum protein, the C1 esterase inhibitor. We showed that danazol corrected the protein abnormality in patients with this disease. During this year we have studied the toxicity of danazol and the effectiveness of the drug in a large patient population. We have determined the minimally effective dose and have defined a whole new series of toxic side effects of danazol. The drug is still considered relatively non-toxic in this patient population at high risk of death from their disease. Acute attacks of hereditary angioedema which do not respond to danazol hav been treated with the intravenous infusion of the purified C1 esterase inhibitive protein and this has stopped the attack within hours of the time of the infusion.

Project Description:

Objectives:

The general objective of this program has been to define the role of complement in human illness and to develop better methods of therapy for immune damage mediated by complement. As in the past year, major emphasis was placed on our studies with patients with hereditary angioedema. This illness, which carries a high mortality rate, is particularly useful for study since it can serve as a model for complement-mediated immune damage in man and as a model for the study of genetically controlled autosomal dominant diseases. We were particularly interested this year in defining the usefulness and toxicity of danazol in a large patient population. We were also interested in extending our efforts to stop attacks of hereditary angioedema after they have already begun. Danazol does not work in this situation.

Methods Employed:

Purified complement components are prepared, and antisera to various complement components and component inhibitors are used for estimation of component levels in human sera and other body fluids. Double-blind therapeutic studies are employed to test new methods of therapy. Patients on experimental drug protocols are followed to determine signs of toxicity.

Major Findings:

In the past years, we have been evaluating the usefulness of the drug danazol in treating hereditary angioedema. Earlier we showed that this drug is markedly effective in treating this disease and is relatively non-toxic. The drug has the striking effect of causing patients with the deficient serum inhibitor protein to synthesize this protein so that levels of the protein in blood return to normal. With the restoration of normal levels of the protein in blood, the patient corrects his biochemical abnormalities and thereafter is free of symptoms. The long-term use of this new drug has never been studied in any patient population and it was essential to begin to define those situations in which the drug was useful or on the other hand was likely to cause problems. We also were interested in attempting to stop attacks of hereditary angioedema once they had begun. It takes days for danazol to have its effect. Patients who are not on danazol however, will often present to an emergency ward having severe angioedema which may be life-threatening. At the present time there is no safe method of treating such patients. The use of fresh, frozen plasma has been advocated, since fresh plasma has the protein which the patients are missing; however, fresh plasma also has complement components in it and the patients are depleted of these components at the time of an attack. It has been suggested that the infusion of complement components will make these patients much more ill before they become better. Thus, fresh plasma is not advocated for use in this disease.

Our findings thus far are as follows. We have treated now in a carefully controlled manner 56 patients with hereditary angioedema. Of this group,

38 are women and 18 are men. The reason that relatively few of the patients are men is that methyltestosterone, which is also useful in treating men, is available and we tend to use this in situations where the patient cannot be followed carefully. Methyltestosterone cannot be used in women as easily as danazol because of its androgenic properties. Its biochemical effects in hereditary angioedema patients are also less striking than those of danazol. This group of 56 patients is an unusual group in that 70% of them had at least one attack per month. Most of our patients have had peripheral swelling attacks, 91% had in addition severe attacks of severe abdominal pain, and 25% had potentially life-threatening oropharyngeal attacks. We began our treatment schedule with 600 mg of danazol. Of the group of patients, 95% responded to 600 mg of danazol with complete relief of clinical symptoms. We then determined the lowest dose of danazol that would be clinically effective. Eighty-eight percent of 56 women responded to 400 mg of danazol, 58% responded to 300 mg of danazol and 11% responded to 200 mg per day of danazol. Thus, most patients respond to 300-400 mg per day, but there is an occasional patient that will respond to 200 mg per day. Side effects of danazol have been studied by very carefully monitoring this patient group. Nine of the patients, or 16%, had abnormal liver function tests. No patient had liver enzymes greater than twice normal, and there was no sign of holestatic jaundice, as occurs with some of the other androgens. Nine patients had mild hematuria without other signs of renal dysfunction. This disappeared when the drug does was decreased and appears to be due to oozing of blood from the bladder. A total of 21 of the 56 patients, that is 38% of the patient group, developed some sign of myopathy. [Seventeen percent of patients had myalgias and cramps, 4% had weakness, and 11 patients, or 20%, had an elevated CPK enzyme level; seven patients, 16%, developed headaches.] Five patients, 13%, had abnormal menses requiring therapy. Five patients, 13%, had decreased libido. Seven patients, 13%, had hair loss. Eighteen patients, or a total of 32%, developed mild to severe anxiety reactions. One patient developed a deepening of her voice, three patients noted a mild increase in body hair, and two patients a decrease in breast tissue. In all, although the list of side effects is impressive, the toxic signs in patients receiving danazol were quite mild, and in fact, only a few patients in this large population group had to be taken off this drug because of these difficulties.

Our infusion studies are also progressing in a highly satisfactory way. Our only difficulty is in getting largw quantities of the C1 esterase inhibitor prepared by the American Red Cross. Basically, we have now completed eight infusion studies in a total of five patients having acute attacks of hereditary angioedema. We have infused the equivalent of about 1,000 cc of whole blood C1 esterase inhibitor into each patient. Every single patient had a positive clinical response, ending his attack of hereditary angioedema over a time span that varied from 30 minutes in three patients, to eight hours in one. It is impossible to control these studies completely; nevertheless, the patients strongly feel that their previous histories of attack are such, that they are certain that the infusion is effective. We have had one or two instances of rebound a day or two after the infusion, with a recrudescence of symptoms. This is of some concern and is undergoing further evaluation at this time.

Proposed Course:

We are continuing to evaluate the use of these drugs in patients with this particular disease. We are also gradually increasing the number of other patients with other complement-related abnormalities which are under study. These include such diverse illnesses as autoimmune hemolytic anemia and systemic lupus erythematosus.

Publications:

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3. Levinson, A.I., Summers, R.J., Lawley, T.J., Evans, R., III, and Frank, M.M.: Evaluation of the adverse effects of long-term hyposensitization. J. Allergy Clin. Immunol. In press.

4. Atkinson, J.P. and Frank, M.M.: The Complement System. In Parker, C. (Ed.): Clinical Immunology. Philadelphia, PA, W. B. Saunders Co. In Press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00051-07 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Host Defense Mechanism Against Pseudomonas Infection in Normal and Immunosuppressed Hosts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.P. Aduan	Medical Officer	LCI	NIAID
OTHER:	E. Harvey	Bio. Lab. Tech. (Micro.)	LCI	NIAID

COOPERATING UNITS (if any)
A.S. Levin, A.B. Deisserroth, and F.R. Applebaum (Pediatric Oncology Branch, NCI, NIH)

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Bacterial Disease Section

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NIAID, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 3/12	1 3/12	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The general areas of pulmonary host defense and nosocomial infection are being investigated, using Pseudomonas aeruginosa infection as the prototype because of its frequent occurrence in immunosuppressed patients. Nosocomial infection is being studied in immunosuppressed animals with emphasis on protection against death by active immunization with a type-specific lipopolysaccharide antigen and a "cross-protective" core antigen, OEP. In particular the efficacy of these vaccines in reducing the quantitative requirements for granulocytes to protect against death in neutropenic hosts is being investigated.

Project Destription:

Objectives:

Pseudomonas aeruginosa is a serious opportunistic bacterial pathogen for patients with impaired host defense mechanisms, particularily patients with hematologic malignancies or patients receiving immunosuppressive therapy for various neoplastic or other systemic diseases. Also, patients with extensive thermal injury or structural lung disease, particularly those who require assisted ventilation, are at high risk to develop Pseudomonas infection. Pseudomonas colonization of the respiratory tract and subsequent pneumonia is associated with a high mortality despite appropriate antibiotic treatment. An important approach to improved treatment of Pseudomonas pulmonary infection is that of immunological prophylaxis through the development of specific anti-Pseudomonas immunity in the serum (humoral antibody) and in the respiratory tract (local antibody and local cell-mediated immunity). Specific antibody may help prevent bacteremia and endotoxemia, and assist the lung's cellular defense mechanism (alveolar macrophages) by enhancing phagocytosis of the Pseudomonas. Cell-mediated immunity to Pseudomonas antigens in the respiratory tract may be of value in "activating" the alveolar macrophages and enhancing both phagocytosis and intracellular killing of Pseudomonas. Currently available Pseudomonas vaccines (lipopolysaccharides) are of limited value because of their toxicity and the presence of multiple immunotypes which lack a cross-reacting antigen. The recent description of a common-protective actigen, OEP (a "core antigen" common to all immunotypes of Pseudomonas) prompted use to investigate the use of this antigen in both humoral and cell-mediated immunity against Pseudomonas in normal and immunosuppressed experimental animals.

Methods Employed:

Animals are vaccinated with a variety of monovalent Pseudomonas vaccines: lipopolysaccharide or "core antigen" - OEP. Hemagglutinating antibodies, total titers and 2-mercaptoethanol resistant (IgG) titers are measured. Isolation and purification of IgG and IgM antibodies is carried out using standard concentration and separation techniques, including ion-exchange chromatography. Protein concentrations are quantitated by radial immunodiffusion precipitation techniques. Alveolar macrophages are obtained from normal and immunosuppressed animals by bronchoalveolar lavage and established in tissue culture monolayers with well-described techniques. Macrophage "activation" is studied in vitro by addition of Pseudomonas products, LPS or OEP, or synthetic adjuvants such as muramyl dipeptide (MDP). Activation of alveolar macrophages by soluble products (lymphokines) of stimulated lymphocytes is studied in vitro by standard techniques. Alveolar macrophage chemotactic factor is obtained by activation of the macrophages by surface contact or phagocytosis of killed staphylococci. Chemotactic factor is purified by gel filtration and assayed by the leading front assay in Boyden chambers. Recruitment of granulocytes into the lung is studied in experimental animals by aerosolization of chemotactic factors into the lung and quantification of different cell types in the bronchoalveolar lavage fluid.

Chemotactic factors derived from bacterial (LPS), serum (complement component (C5a), or synthetic peptides (formyl-methionine peptides) are used. Nosocomial Pseudomonas infection is studied in an established leukopenic dog model. Dogs are rendered leukopenic by total body gamma-irradiation or intravenous cyclophospamide and challenged with intra-bronchial instillation or intravenous injection of a lethal number of Pseudomonas bacteria.

Major Findings:

The antibody response of canines to the Pseudomonas lipopolysaccharide (LPS) and "core-antigen" (OEP) has been defined. Antibody against these antigens have been purified. We have successfully defined a cytoxan induced leukopenic dog model for studying Pseudomonas bacteremia in the compromised host. We have investigated the efficacy of active immunization and granulocyte transfusions in prolonging the survival of animals challenged with a lethal dose of Pseudomonas. The major factor influencing survival was not the immune status of the animal but, rather the level and duration of granulocytopenia. High antibody titers to each of the pseudomonas antigens studied were ineffective in the absence of an adequate leukocyte level. Current analysis of the data is directed at attempting to determine whether the antibody augments the protection offered by white cell transfusions.

Significance to Biomedical Research and the Program of the Institute:

Pseudomonas bacteremia and penumonia are commonly encountered in compromised and immunosuppressed patients. The mortality from these infections remains high despite recent advances in antibiotic therapy and patient management. Methods of restoring or enhancing impaired host defense mechanisms must be sought. Although granulocyte transfusion therapy has proven efficacious in experimental trials, it may not have practical utility because of technical and monetary problems. Immunoprophylaxis, in the form of enhanced humoral and cell-mediated immune mechanisms, and non-specific "activation" of phagocytic cells (macrophages) by adjuvants, are two approaches to improving the management of infection in compromised hosts. The combination of immunoprophlyaxis and restoration of host defenses by granulocyte transfusion therapy may be necessary in order to improve survival in patients with Pseudomonas bacterimia and pneumonia. It is possible that non-specific and specific immunoprophylaxis may reduce quantitative requirements for granulocyte replacement.

Recent investigations of "core-antigens" (Lipid A) as vaccines in immunoprophylaxis against gram-negative organisms have suggested that broad protection may be afforded by a single vaccine. However, Pseudomonas does not share this common antigen with other members of the Enterobacteriacea. The "core-antigen" of Pseudomonas (OEP) may prove to be of great importance because of its common protective capabilities (among the multiple immunotypes of Pseudomonas), its non-specific adjuvant effects, and its lesser toxicity (as compared to lipopolysaccharides).

Proposed Course:

This project has been terminated because the principal investigator has transferred to the University of Washington, Seattle.

Publications:

1. Aduan, R.P. and Reynolds, H.Y.: The importance of cell-mediated responses to Pseudomonas infection. In Doggett, R.G. (Ed.): Pseudomonas Aeruginosa: Clinical Manifestations of Infection and Current Therapy. New York, Academic Press. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00054-06 LCI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Etiology and Pathogenesis of Viral Gastrointestinal and Respiratory Tract Infections in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The etiology and pathogenesis of viral gastrointestinal and respiratory tract infections in man are being examined. A virus particle morphologically similar to but antigenically distinct from the Norwalk agent has been associated with a waterborne outbreak of gastroenteritis, and the disease has been transmitted to normal volunteers. Cell-mediated immune studies of influenza infection and vaccination in man indicate that both antigenically specific and nonspecific cytotoxic responses are seen. Patients with systemic lupus lack cytotoxic responses after vaccination and have variable humoral antibody responses. Clinical trials of monovalent and trivalent inactivated vaccines containing influenza A/USSR/90/77 (H1N1) antigens in volunteers, primarily over 24 years of age, resulted in low reactogenicity and high rates of sero-responses.

Project Description:

Objectives:

1) To investigate the pathogenesis of and host response to viral infections of the respiratory and gastrointestinal tracts in man. Experimentally induced disease in normal volunteers, as well as naturally occurring cases are studied.

2) To define the biologic, immunologic, and epidemiologic properties of the etiologic agents of viral gastroenteritis in man.

3) To develop in vitro culture systems and/or laboratory animal models with which to study these agents.

4) To examine the possible roles of viral agents in acute and chronic gastrointestinal and respiratory tract diseases of unknown etiology.

5) To devise control measures based on the above information, with particular emphasis on the use of antiviral compounds.

Methods Employed:

1) Source of Viral Agents: Viral agents have been obtained from naturally occurring outbreaks of viral gastroenteritis and have been partially characterized. These are maintained as infectious suspensions in veal infusion broth and serve as infectious inocula and sources of antigen. Respiratory tract viruses are isolated in tissue and organ culture from naturally occurring cases. After characterization, they are grown in large quantities to serve as standard sources of the agent for laboratory and volunteer studies.

2) In Vitro Culture Systems: Standard tissue cultures or suckling mice are employed for the isolation of conventional viral agents. Since certain fastidious agents replicate only in organ culture, human fetal intestinal and tracheal organ cultures are employed for the detection of new or occult agents. The organ cultures are examined with light, fluorescence and electron microscopy, and the explants are tested for viral interference, metabolic alterations, and enzyme production.

3) Clinical Studies

a) Volunteer Studies: After rigorous safety tests of the inocula, normal volunteers are screened for appropriate serum antibody status and are infected via oral or nasopharyngeal administration of these viruses. The volunteers are followed closely for clinical changes, and samples of blood, stool, respiratory secretions, along with selected biopsies, are obtained.

b) Naturally Occurring Cases: Similar studies are performed on outpatients and hospitalized patients referred by local physicians or the

infectious disease consultation service of the LCI. Lung biopsies of acute interstitial pulmonary infiltrates in host defense-compromised patients hospitalized at the Clinical Center are provided through the consultation service of the LCI.

4) Host-Defense Studies: Serum and secretory antibody responses to viral infection are examined, employing neutralization, complement fixation, hemagglutination inhibition or fluorescent antibody assays. Cell-mediated immunity is investigated by mitogenic and antigenic stimulation of lymphocyte cultures and by lymphocytotoxicity assays against virus-infected target cells. Lymphocyte subpopulations are determined by sheep red blood cell and complement receptor assays (Fauci). Nonspecific defenses such as polymorphonuclear leukocyte functions and interferon production are also examined.

Major Findings:

1) Detection of New Agent of Viral Gastroenteritis

a) A 27 to 32 nm particle with cubic symmetry was detected by immune electron microscopy (Dolin, Tralka) in two of five diarrheal stools provided by the CDC (Morens, Zweighaft) from an outbreak of gastroenteritis at Granby, Colorado, in which the agent appeared to be waterborne, and person-to-person spread was documented. Administration of bacteria-free filtrates to two normal volunteers resulted in induction of illness consisting of nausea, vomiting, diarrhea, and low-grade fever, with an incubation period of 24 to 36 hours. Viral-like particles were also observed in the stools of one of the ill volunteers. The particles appear similar to the Norwalk agent but are antigenically distinct by immune electron microscopy. Additional characterization studies are underway. This agent, referred to as the "Colorado agent", represents the first documented waterborne agent of viral gastroenteritis and suggests that waterborne reservoirs exist for this illness.

b) Inocula containing the Norwalk agent (8 FIIa) were administered to normal volunteers in increasing dilutions, 10^{-2} and 10^{-3}. Illness, indistinguishable from that observed with undiluted inocula, was induced, indicating that at least $10^{3.5}$ human disease-producing doses were present and that induction of illness was not dose dependent. Administration of undiluted 8FIIa represents a high titered challenge, so that "overwhelming" of naturally acquired immunity may occur. Additional dilutions will be studied over the upcoming year to determine a minimum disease-producing dose with which to investigate immunity in challenge studies.

2) Cell-Mediated Immunity in Live and Inactivated Influenza Vaccination and in Naturally Occurring Influenza

a) In previous studies, we described a cell-mediated cytotoxic response after experimental infection of volunteers with wild type influenza A viruses. This response was broadly directed at homologous and heterologous ortho- and paramyxoviruses. We performed similar studies employing a ^{51}Cr

release assay with MA 160 tissue culture cells infected with a variety of viruses in volunteers after vaccination with inactivated influenza A/Victoria/3/75 (H3N2) vaccine. Elevated cytotoxicity developed by day 4 after vaccination and returned to base line at day 8. Cytotoxicity was directed at influenza A but not B-infected targets. This cytotoxicity appeared to be restricted to antigenically related viruses, in contrast to that seen after infection. Fractionation of peripheral blood mononuclear cells indicates that effector cells have the properties of non-T lymphocytes and can only partially be eliminated by depletion of adherent cells. Antibody has not been detected in our assay system, although small amounts cannot be excluded as playing a role. Thus, influenza infection appears to stimulate both antigen specific and nonspecific killing. The kinetics of the response suggest that multiple effector cell systems may be activated.

b) Studies of cell-mediated cytotoxicity in 14 patients with systemic lupus erythematosus have demonstrated decreased cytotoxicity directed at target cells infected with influenza A, B, Newcastle disease virus, and herpes simplex virus. In contrast to matched normal subjects, SLE patients fail to generate cytotoxicity following influenza immunization, although humoral antibody responses developed. This depressed cytotoxicity appears unrelated to disease activity or to steroid administration. In fact, administration of 400 mg of hydrocortisone IV partially reversed this defect in cytotoxicity in four patients.

c) Studies of cell-mediated immunity in vaccination of normal volunteers with live, intranasally administered, temperature sensitive influenza viruses failed to stimulate cytotoxic responses but stimulated a modest amount of blastogenic responses to purified influenza antigens. In contrast, naturally occurring infection with influenza A/USSR/77 (H1N1) resulted in markedly elevated levels of cytotoxicity and blastogenesis. Thus, a spectrum of CMI responses has been observed, from little or no responses with ts viruses, to modest responses with experimentally induced illness to markedly elevated responses with naturally occurring disease.

3) Clinical Trials With Inactivated Influenza Vaccines

One hundred and eight volunteers, between 13 and 64 years of age, were studied in clinical trials of inactivated influenza vaccines. Monovalent [A/USSR/90/77(H1N1)] vaccine was administered, followed four weeks later by trivalent vaccine which contained A/USSR/90/77(H1N1), A/Texas/77 (H3N2) and B/HK/72 antigens. Significant reactions were infrequent (<5%) and high rates of sero-conversion were seen (generally >80%) to A/USSR after a single dose. Similar influenza vaccine trials in SLE patients, indicated that individuals with pre-existing antibody had high rates of sero-conversion, but "unprimed" individuals had a poor response.

4) Studies of Naturally Occurring Influenza at University of Maryland College Park Campus

In February, 1978, an explosive and widespread outbreak of influenza

A/USSR/77(H1N1) occurred at the University of Maryland College Park Campus and was investigated by virus isolation, serology, clinical questionnaire, and outpatient chart review. A/USSR virus was isolated from 9/12 acutely ill patients, all of whom manifested fourfold or greater rises in HAI antibody. Clinical attack rates from 670 students were approximately 70% and were higher in those ≤23 years of age and in those who lived on campus. Serology on 190 students showed rates of infection approaching 90% in those ≤23 years old. Illness was generally mild, although prolonged in 18% of cases. These findings suggest that only prophylactic or rapidly acting control measures are likely to be effective in this setting.

5) Clinical Virology

Two hundred and forty-eight specimens were examined for viral isolations in support of the infectious disease consultation service of the LCI or at the request of physicians at the NIH, NNMC or in the community. Ninety-four were positive for a wide variety of viral agents. Selected viral serologies were also performed to confirm diagnoses of viral infection.

Significance to Biomedical Research and the Program of the Institute:

Viral respiratory and gastrointestinal tract infections are the most common disease experiences in American families and affect all age groups of a broad segment of the population. As yet, there are no adequate control measures available. Identification and characterization of etiologic agents, as well as studies of the pathogenesis of these diseases in man, should form the basis for rational development of methods of prevention and treatment. The newly developed techniques described above, along with volunteer experiments should continue to provide valuable data.

Proposed Course:

The pathogenesis and host response of both respiratory tract and gastrointestinal viral infections in man will continue to be examined in both experimentally induced and naturally occurring disease. The newly developed techniques for measuring cell-mediated immune responses to influenza antigens will be applied to the study of complications of naturally occurring viral disease, such as overwhelming pneumonia. Prospective studies of SLE patients for susceptibility to viral infections will be undertaken to evaluate the role of defective CMI responses in various viral infections. Rapid diagnostic techniques for detection of viral antigens by enzyme-linked immunoadsorbant assays are being developed. Studies of the agents of viral gastroenteritis will continue with emphasis on propagation of agents in vitro, and on the development of rapid sensitive detection methods, with particular emphasis on characterization of the "Colorado agent" and its antigenic relationship to the parvovirus-like agents. The potential application of antiviral compounds to the prophylaxis and/or therapy of viral respiratory and gastrointestinal disease will be investigated.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00055-06-LCI

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October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Regulation of the Immune Response in Man and Experimental Animals

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SUMMARY OF WORK (200 words or less - underline keywords) Utilizing a highly sensitive in vitro plaque forming cell assay which was developed in this laboratory to study human B lymphocyte function, we have delineated several of the complex mechanisms of immunoregulation in normal immunologic reactivity and in diseases characterized by abberancies of immunoregulation. We have described the mechanisms and events associated with B cell triggering and subsequent differentiation and production of antibody. We have characterized the kinetics, mechanisms, and subpopulations involved in the regulation of B cell function in the normal immune response. In addition, we have demonstrated that normal immune reactivity is the net result of a balance between positive and negative immunoregulatory influences mediated directly or indirectly by distinct subpopulations of cells. In diseases characterized by abnormalities of immunoregulation, we have related several abnormalities of immune function directly or indirectly to the increase or deficiency of various regulatory cell subpopulations, abnormalities in their functional capabilities, as well as to the state of their activation. The differential sensitivity of these various parameters to a variety of clinically relevant manipulations such as corticosteroid therapy has been described.

Project Description

Objectives:

1) To delineate the mechanisms of regulation of immunologic reactivity by purified subpopulations of lymphoid cells predominantly in human peripheral blood. To examine the precise functional capabilities of these individual lymphoid cell subpopulations as well as the mechanisms whereby they maintain the normal homeostasis of positive and negative signals which regulate normal immunologic reactivity.

2) To determine the role of these lymphoid cell subpopulations in the pathogenesis of immunologically mediated diseases such as hypersensitivity diseases, granulomatous diseases, the spectrum of autoimmune diseases, as well as infectious diseases characterized by aberrations of immunologic reactivity. In particular, to determine the mechanisms whereby alterations in various subpopulations of cells, for example, by abnormal activation by immune complexes or viruses, by depletion, proliferation, or redistribution among lymphoid compartments result in abnormalities of immunoregulation characteristic of these diseases.

3) To investigate the cellular interactions, molecular, and biochemical events associated with triggering of various mononuclear cell subpopulations and to determine the relationship between selective triggering via various cell surface receptors and the kinetics of expression of various functional capabilities of the given cell populations.

4) To develop an *in vitro* model of autoreactivity by selective triggering or depletion of immunoregulatory cell subpopulations in order to define more precisely the primary or secondary role of altered immunoregulation in autoimmune diseases.

5) To continue to develop and further perfect culture and assay systems for primary *in vitro* activation of human B lymphocytes to antibody production following polyclonal activation or specific antigenic stimulation.

6) To induce, purify and characterize soluble factors from purified subpopulations of human lymphocytes and monocytes which are involved in the regulation of immunologic reactivity.

7) To delineate the precise mechanisms whereby immunosuppressive agents such as corticosteroids and cytotoxic drugs affect the immune response in man specifically examining their selective effects on subpopulations of lymphocytes and monocytes with regard to their circulatory kinetics, cellular interactions, activation, and expression of *in vivo* and *in vitro* functional capabilities. In addition, to correlate the selective effects of corticosteroids and cytotoxic drugs on these various parameters with the suppression of disease activity in various inflammatory and immunogically-mediated disease states in man and by doing so to aim at a greater understanding and therapeutic specificity in the clinical use of these agents. Furthermore, to study the mechanisms whereby manipulations of immune reactivity as described above

affect host defenses against infection, tumor surveillance and propensity towards auto-immune states.

8) To study the functional capabilities of eosinophils and mononuclear cells in the idiopathic hypereosinophilic syndrome in order to elucidate the mechanisms whereby these cells cause invasion of tissue and subsequent tissue damage in this disease. To use this information to gain a greater understanding of the functional properties of eosinophils in normal states, various diseases and to determine their sensitivity to various therapeutic regimens.

9) To extend the concept of immunoregulation to the effect of the immune system on other cell types. Specifically, to study the mechanisms of immunoregulation by lymphoid cell subpopulations on eosinophil and neutrophil kinetics and function in the hypereosinophilic syndrome and certain neutropenic states respectively.

10) To continue to study the spectrum of the vasculitides in man from a mechanistic, pathophysiologic, clinical, and therapeutic standpoint.

Methods Employed:

1) A major component of the methodology relates to the fractionation, identification and purification of lymphoid cell subpopulations and the in vitro culturing of these cells to determine their diverse functional capabilities. Subpopulations of cells are identified predmoninantly by surface markers and functional capabilities are assessed by a variety of in vitro assays. We have focused on the relationship between various in vivo and in vitro triggering signals and the subsequent in vivo expression of direct and immunoregulatory functional capabilities. This has been focused predominantly in the area of activation, proliferation and differentiation of human B lymphocytes to antibody producing and secreting cells. In this regard, we have been utilizing a unique model of B cell function which was originally developed in this laboratory. It is a system of primary in vitro stimulation of bone marrow derived (B) lymphocytes by polyclonal activation as well as specific antigenic stimulation with subsequent measurement of single cell antibody production by a direct hemolysis-in-gel plaque forming cell assay. Using this model, we have developed a system for the selective generation of suppressor cells or helper cells capable of modulating in vitro antibody production. In addition we have produced soluble mediators of B cell function in man and have characterized the cell types of their origin.

Other assays employed to delineate the functional capabilities of lymphoid cells are the in vitro blastogenic responses to mitogenic and antigenic stimulation, elaboration of various soluble mediators, cell mediated cytotoxicity against various autologous, allogeneic and xenogeneic targets. Various cytotoxicity assays include spontaneous, mitogen dependent, mitogen generated, and antibody dependent cellular cytotoxicity. A particular aim is to identify and characterize the lymphocyte or monocyte subpopulations involved in immunologically mediated diseases as well as to create in vitro models of altered immunoregulation by selectively triggering or manipulating immunoregulatory subpopulations of cells.

2) Corticosteroids are administered to normal volunteers and patients requiring this drug and selective effects of these agents on the compartmentalization and functional capabilities of monocytes and lymphocyte subpopulations are determined. In addition, the differential effects of in vitro corticosteroids and in vitro irradiation on various lymphoid cell functional capabilities such as triggering, proliferation, differentiation, elaboration of mediators, suppressor cell and/or helper cell function, antibody production and secretion are being studied.

3) A subject's lymphoid cells are labeled with ^{51}chromium and reinfused back into their circulation to determine the effects of disease states and of chemotherapeutic agents on the viability and circulation patterns of these cells.

4) Bone marrow aspirates are performed in normal subjects and patients with various diseases prior to, during and following therapy. Purified populations of lymphoid cells are obtained from these bone marrow cell suspensions by various separation techniques in order to delineate the immunocompetence of bone marrow lymphoid cells in normal man, as well as the alteration of compartmentalization and function of these cells in untreated and treated disease states.

5) In addition, we are developing antisera against purified subpopulations of lymphoid cells in order to selectively inhibit their functional capabilities or deplete them from various cell suspensions. In conjunction with this we are developing continuous cell lines derived from purified subpopulations of lymphocytes.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) The most significant series of developments in this laboratory over the past year with important implications to biomedical research have been the series of studies which have delineated several of the mechanisms of immunoregulation of human B cell function in the normal immune response and in several diseases characterized by aberrations in immunologic reactivity. To this end, we have utilized and extended the unique assay of human B cell function originally developed in this laboratory. It is a system of primary in vitro stimulation of B lymphocytes by polyclonal B cell activators or antigens, with measurement of single cell antibody production by a direct hemolysis-in-gel plaque forming cell assay. Representatives of numerous laboratories throughout the country and the world have visited our laboratory to learn this methodologic approach and have now successfully instituted it in their own laboratories.

2) Significant findings from this laboratory over the past year in the area of immunoregulation of human B cell function have been:

a) The demonstration among a percentage of normal individuals of a non-responder state of B lymphocytes to polyclonally induced anti-sheep red blood cell plaque forming cell induction, and the demonstration that

this non-responder state results from the presence of active suppression mediated by identifiable subpopulations of lymphoid cells.

b) Extension of the studies on the mitogen-induced generation of suppressor cells of B cell function in which the subpopulations of suppressor cells have been identified and characterized. In addition, the kinetics and mechanisms of these suppressor cells have been delineated.

c) Demonstration of the selective effect of corticosteroids, irradiation and cyclic AMP on subpopulations of immunoregulatory cells.

d) The in vitro induction and elaboration of soluble regulatory factors of human B cell function by subsets of mononuclear cells.

e) The demonstration of altered proportions and absolute numbers of immunoregulatory T cell subpopulations in patients with systemic lupus erythematosus (SLE). In addition, the demonstration of a deficiency of suppressor cell function in SLE patients. In this regard, an in vitro model of pre-triggering suppressor cells by activation with immune complexes has been developed to support the hypothesis of immune complex associated alterations of immunoregulation in SLE.

f) The demonstration of altered proportions and absolute numbers of immunoregulatory T cell subpopulations in patients with sarcoidosis, as well as the demonstration of the presence of abnormal suppressor monocytes in this disease.

g) The demonstration of the appearance and disappearance of suppressor cells of B cell function in acute and convalescent infectious mononucleosis respectively.

3) We have further extended our previous studies on the selective effects of in vivo administration of corticosteroids on various subpopulations of lymphoid cells. In addition to the demonstration that T lymphocytes are more sensitive to the lymphodepletive effects of corticosteroids than are non-T lymphocytes, we have recently demonstrated that within the T cell population, different subsets of cells are selectively affected by the administration of the hormone. T cells with an Fc receptor for IgM are markedly depleted from the circulation following administration of corticosteroids, while T cells with an Fc receptor for IgG are relatively resistant to these lymphodepletive effects.

4) We have demonstrated that influenza A infection experimentally induced in normal volunteers results in an unresponsive state of circulating B cells to polyclonal triggering, most likely due to the activation of suppressor cell subpopulations.

7) We are studying a large group of patients with various categories of systemic vasculitis. Based on observations related to mechanistic features

clinicopathologic manifestations, and therapeutic responses, we have developed a revised classification of the entire spectrum of the systemic vasculitides.

In this regard, we have continued and extended our studies of the effects of corticosteroids and cytotoxic agents in vasculitic and granulomatous diseases. Previously, we have described impressive results with cyclophosphamide in Wegener's granulomatosis (WG). Prior to the publication of the clinical studies of our patients, WG was a uniformly fatal disease. We have essentially effected long term remissions and cures of this disease with cyclophosphamide therapy. Our results are now being confirmed throughout the country and the world. Because of these encouraging results, we have now begun using cyclophosphamide in the polyarteritis nodosa group of systemic necrotizing vasculitis and in corticosteroid resistant disseminated vasculitis. This past year, we have reported our remarkably favorable results with the use of these therapeutic modalities in the treatment of the systemic vasculitides.

In addition to serving as excellent study models of hypersensitivity diseases and the effect of cytotoxic therapy on such disease states, our experience with these disorders has allowed us to already make, and to continue to make several important and original observations on the effect of low dose cytotoxic therapy on subpopulations of lymphoid cells in man.

In addition to our successes with these diseases, we are continuing to demonstrate remarkable therapeutic successes with radiation therapy in midline granuloma.

6) We continue to follow the largest single group of patients with the idiopathic hypereosinophilic syndrome in the world. We have developed guidelines for the treatment of this disorder with corticosteroids and/or hydroxyurea which are now being employed at several major centers. We have achieved greater than 90% remission in this disease with our therapeutic regimen.

In addition, we have employed a unique fractionation procedure developed in this laboratory to obtain purified populations of eosinophils. Using this technique we have characterized the surface markers as well as various functional properties of these cells. Moreover, we have most recently delineated the immunologic abnormalities associated with the hypereosinophilic syndrome.

Proposed Course:

This project will continue along the lines which have been described.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00056-06 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Biochemical Pathways of Mediator Release and Mechanism of Tissue Injury in Allergic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.P. Kaplan	Head, Allergic Diseases Section	LCI NIAID
OTHER:	M.A. Kaliner	Senior Investigator	LCI NIAID
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5 7/12	3 7/12	2

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) High Molecular Weight (HMW)-kininogen was shown to form a 1:1 stoichiometric complex with either plasma prekallikrein or factor XI and the light chain of HMW-kininogen was shown to be responsible for this interaction. In this fashion HMW-kininogen functions as a plasma cofactor required for coagulation, fibrinolysis and kinin-formation. Activation of factor XI to factor XIa was shown to occur by limited proteolysis in which two 80,000 dalton disulfide-linked chains are each digested within intrchain disulfide bonds to yield 50,000 and 30,000 dalton disulfide linked fragments. Factor XIa was shown to directly convert plasminogen to plasmin and appeared to be equipotent with kallikrein as a plasmenogen activator. Histamine was shown to recruit eosinophils into human skin however allergic individuals had a markedly heightened eosinophil response compared to non-atopics. A case of combined cold and cholinergic urticaria was successfully treated with atarax plus periactin and the time course of histamine release was contrasted for each type of challenge. Cyproheptadine was shown to inhibit the effects of histamine in vivo in cold urticaria but had no effect upon the release of histamine. Thus cold challenge of treated patients yielded brisk histamine release but no symptoms and cyproheptadine acts in vivo as a potent H-1 type histamine antagonist.

Project Description:

Objectives:

1) Determination of the role of prekallikrein factor XI and high molecular weight kininogen (HMW kininogen) in the Hageman factor dependent pathways of coagulation and fibrinolysis and kinin formation. Methods for isolating each component will be developed, and its mechanism of activation dependent pathways will be assessed. Radioimmunoassays or radial immunoplasma will be developed focusing attention upon the early steps of the intrinsic coagulation and fibrinolytic pathways.

2) Determine the mechanism by which the Hageman factor dependent and Hageman factor independent fibrinolysis occurs when human plasma is activated.

3) Study the mechanism by which negatively charged surfaces interact with Hageman factor, prekallikrein and HMW kininogen to expose an active site which can initiate coagulation, fibrinolysis, and kinin formation.

4) Further investigation of the ability of histamine to function as a chemotactic factor for human eosinophils and to modulate the response of eosinophils to other agents. The response of eosinophils to histamine in vivo will be compared to in vitro observations using the Boyden chamber.

5) Further investigation of the pathogenesis of cold urticaria, dermographism, cholinergic urticaria, and chronic idiopathic urticaria and angiodema. Attention will focus upon an identification of the vasoactive principles that cause the symptoms, identifying any immunologic mechanism that may play a role, and defining the biochemical pathway that is activated in each disorder.

Particular attention will be given to the role played by IgE antibody, the human basophil and mast cell, and the release of prostaglandins and kinins in the skin.

6) Determination of the recruitment of non-basophil or mast cell inflammatory mediators as a consequence of IgE mediated reactions such as an insect sting anaphylaxis.

Methods Employed:

Purification of plasma proteins is performed utilizing affinity chromatography, ion exchange chromatography on Quarternary aminoethyl (QAE) Sephadex and Sulphopropyl (SP) Sephadex, gel filtration, and preparative elution from gels after isoelectric focusing. These methods have been applied to the isolation of unactivated Hageman factor, Hageman factor prealbumin fragments, pre-PTA (coagulation Factor XI), prekallikrein, plasminogen and the plasma inhibitors α_1 antitrypsin, α_2 macroglobulin, the inhibitor of the activated first component of complement (CI INH), inter α trypsin inhibitor,

α_1 chymotrypsin inhibitor, and antithrombin III. Purity is evaluated by alkaline disc gel electrophoresis, SDS gel electrophoresis, and isoelectric focusing.

Functional assays routinely utilized were correction of partial thromboplastin time of congenitally deficient plasma to assay for Hageman factor, factor XI, prekallikrein, and HMW kininogen.

For kinin assays, bradykinin was measured by bioassay utilizing the guinea pig ileum. Kallikrein was assayed by its ability to generate bradykinin from HMW kininogen. Prekallikrein was assayed by activation with Hageman factor fragments and the kallikrein generated determined. Kallikrein could also be assayed quantitatively by cleavage of the synthetic substrate α-Benzoyl-pro-phe-arg-p-nitroanilide to release p-nitroaniline. Activated Hageman factor or factor XI_A were assayed by their ability to clot deficient plasma in the absence of kaolin and HF_f were assayed by their ability to generate bradykinin in EDTA-plasma.

Plasmin was assayed in fibrin plates or by digestion of H-D-Val-leu-Lys-p-nitroanilide. Plasminogen activators were assayed by incubation with excess plasminogen and the plasmin generated was determined.

Histamine and serotonin were determined by radioenzyme assays.

Suction blisters were induced using mild heat and negative pressure to yield a 1 cm. subepidermal blister on the forearm of patients and volunteers. Passive transfer studies were performed by injected sera intradermally, waiting 4 hours to allow IgE to bind to mast cells and other immunoglobulins to diffuse away, followed by challenge of the site. The ability of histamine to recruit eosinophils into human skin sites was studied using the skin window technique. Solutions of histamine were applied by soaking cotton with the solution enclosed by a chamber with a millipore filter at one end. The filter was placed over the abraded area and changed at intervals at over a 24 hour period. Cells that migrated into the filter were counted at each time interval.

Major Findings:

1) Both factor XI and prekallikrein were found to circulate in plasma as a complex with high molecular weight (HMW) kininogen. They were shown to bind as 1:1 molar complexes; however a molecule of HMW-kininogen could bind to a molecule of either prekallikrein in factor XI but not both. The activation of Factor XI was dependent upon the interaction of Hageman factor with the prekallikrein-HMW kininogen complex; activated Hageman factor converts factor XI to factor XI_a. It was proposed that kallikrein may migrate along the surface to activate Hageman factor molecules in proximity to factor XI.

2) The structural features that render HMW-kininogen a coagulation factor were further investigated. HMW-kininogen was found to be a single chain of molecular weight 120,000 upon SDS gel electrophoresis and no change in molecular weight reduction was seen. Upon digestion with plasma kallikrein, bradykinin was liberated by two internal cleavages within a disulfide

bridge such that the kinin free molecule consisted of a two chain structure with a heavy chain of approximately 70,000 disulfide linked to a light chain of 37,000. When the chains were separated by gel filtration, all of the coagulant activity resided in the light chain.

The purified light chain was then tested for its ability to interact with both prekallikrein and factor XI. When polypropylene tubes were coated with light chain, specific binding of either ^{125}I-prekallikrein or ^{125}I-factor XI to the light chain was observed. No binding to the heavy chain was seen when tested under identical condition. The binding constant for the interaction of factor XI and the light chain was 10^6 mole^{-1}. In addition the isolated light chain was shown to compete with native HMW-kininogen for binding prekallikrein and factor XI. Thus the cofactor function of HMW-kininogen was shown to be a property of the light chain; it accomplishes this by binding the Hageman factor substrates in plasma and then binding them onto negatively charged surfaces where the interaction with Hageman factor proceeds.

3) Factor XI was purified and shown to be a two-chain structure in its zymogen form. Two apparently identical chains are linked by disulfide bonds thus its molecular weight changes from 160,000 to 80,000 upon reduction. Upon activation to factor XIa, no change in molecular weight was seen in the absence of reduction i.e. it remained at 160,000. However, reduction of factor XIa yielded two chains of 50,000 and 30,000. Thus each 80,000 dalton chain was cleaved to a heavy chain of 50,000 daltons which was disulfide linked to a light chain of 30,000 daltons. The active site of the enzyme was found to reside in the light chain and the data suggest that factor XIa may contain two identical active sites per mole because of its two-chain structure. Factor XIa was found to directly activate plasminogen and its ability of kallikrein to activate plasminogen. Factor XIa and kallikrein appeared to have the same potency as plasminogen activators however there are four times more kallikrein that can be generated in plasma than factor XIa. Thus kallikrein may be the major factor. However, factor XIa may be responsible for the gradual conversion of plasminogen to plasmin that is seen upon surface activation of prekallikrein deficient plasma.

4) Hageman factor was purified by elution from QAE-sephadex using a salt gradient, passage over lysine-sepharose to remove plasminogen, 60 per cent ammonium sulfate precipitation, elution from QAE-Sephadex using a pH gradient, and sequential fractionation on SP Sephadex C-50 utilizing a salt gradient initially, followed by a pH gradient from 5.5 to 8.1 in a Tris-maleate buffer system. The isolaged molecule was 80,000 daltons, could bind to negatively charged surfaces, and was cleaved by kallikrein, factor XIa or plasmin to yield two activated forms. One was a molecule internally digested within a disulfide bond to yield a 50,000 dalton fragment disulfide linked to a 30,000 dalton fragment. The second was cleavage of the molecule into two pieces with formation of the 28,000 dalton Hageman factor fragments. No active site formation in Hageman factor was observed upon binding to the surface in the presence or absence of HMW-kininogen. However addition of prekallikrein (or kallikrein) led to rapid digestion of Hageman factor.

Thus Hageman factor and prekallikrein activate each other in a reciprocal fashion in which HMW-kininogen functions as a cofactor.

5) Mediator release in a variety of forms of urticaria was investigated utilizing induced blisters over urticarial lesions and control skin sites. Such a method for assessment of skin inflammation is needed for those disorders in which plasma mediator levels are normal. Blisters were formed by a combination of heat (47°C) and suction (wall vacuum) applied to chambers attached to the skin with a 1 cm opening at the skin-contact surface. A 1 cm sub-epiderminal blister forms in 20-45 minutes, the fluid aspirated, and assayed. Normals had 0.5-16 ng/ml histamine in their blister fluid with a mean of 7 ug/ml. Six patients with cold urticaria had control blister fluid histamines with a mean of 9 ug/ml while blister fluids over a hive induced by application of an ice cube to the skin averaged over 40 ng/ml. Elevated blister fluid histamines were also demonstrable in solar urticaria, local heart urticaria, and immediate pressure urticaria. The last two disorders had equivalent blister fluid histamines over control and induced sites because the heat and pressure of the apparatus itself induced a lesion. Patients with chronic idiopathic urticaria, for which no etiology is evident, were shown for the first time to have an abnormality in dermal histamine release. Blister fluid histamines, as a group, were over eight times normal; in 1/3 of patients elevated blister fluid histamines were also obtained over normal appearing skin sites suggesting inflammation at the site prior to the formation of visible lesions or an abnormality of skin mast cells which are degranulating in an abnormal fashion secondary to the procedure.

6) The ability of histamine to function as a chemotactic factor in vivo was studied. Upon application to skin windows utilizing chambers containing cotton soaked in histamine, selective migration of eosinophils into human skin was observed. However, a dramatic influx of eosinophils into the skin of atopic individuals was observed and little or no migration of eosinophils was seen when non-atopic individuals were studied. There was no correlation between the eosinophil migration and the eosinophil count. Thus allergic individuals appear to possess enhanced eosinophil responsiveness to histamine.

7) A case of combined cold urticaria and cholinergic urticaria was studied (the fourth ever reported). In each instance histamine release upon either cold or exercise challenge was observed, however the time course of histamine release was different. With cold challenge, a rapid rise and fall of plasma histamine was observed while a slow rise and sustained elevation of plasma histamine was seen upon exercise challenge. Combined therapy with hydroxazine and cyproheptadine resulted in markedly diminished sensitivity to both temperature change and exercise.

8) The mode of action of cyproheptadine in cold urticaria was studied. The patients hand was immersed in ice water for 5 minutes and plasma histamine levels were measured over the ensuing 30 minutes before and after therapy with periactin. Although no puritis or swelling was observed after treatment, histamine release secondary to cold challenge was identical in

each instance. Thus cyproheptadine acts to antagonize the effects of histamine, but does not interfere with the release of histamine.

Proposed Course:

Project to be discontinued.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00057-05 LCI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Basic Studies on Pathogenic Fungi

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.J. Kwon-Chung	Research Microbiologist	LCI NIAID
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T.S. Theodore (LSD, NIAID, NIH)
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 3/12	1 3/12	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to cover basic and applied aspects of various pathogenic fungi including their morphology, taxonomy, pathology, epidemiology, biochemistry, and genetics. The topics of present interest include: 1) biochemical differences among four serotypes of cryptococcus neoformans; and 2) structure of the capsular polysaccharide of serotype C.

Project Description:

Objectives:

Objectives of the project cover both basic and applied aspects of various pathogenic fungi, including their morphology, biochemistry, taxonomy, pathogenicity, life cycle and genetics.

Methods Employed:

1) Production of capsular polysaccharide was carried out by using serotype C. The isolates were grown in glucose neopeptone broth for one week at 25C. Cells were killed by formalin and the isolation of polysaccharide from the supernatant was carried on by precipitating the polysaccharide with 95% ethanol. The polysaccharide was dissolved in distilled water and deproteinated with chloroform and n-butanol. The polysaccharide was precipitated by 95% ethanol, and this procedure was repeated. The polysaccharide was redissolved in distilled water and dialyzed against distilled water with tolvene at 4°C. The polysaccharide was filter-sterilized and lyophilized.

2) Purification and structural study of capsular polysaccharide: The following methods were used: DEAE--cellulose column for chromatography, gel filtration and ultracentrifugation, paper chromatography, hydrolysis of the polysaccharide, methylation, periodate oxidation, chromium trioxide oxidation, measurement of optical rotations, and preparation of carboxyl reduced polysaccharide.

3) Experiments on creatinine utilization. Utilization of creatinine, release of ammonia and change of pH as a result were measured by conventional biochemical methods in four serotypes of C. neoformans.

Major Findings:

1) Utilization of Creatinine by the Four Serotypes of Cryptococcus neoformans.

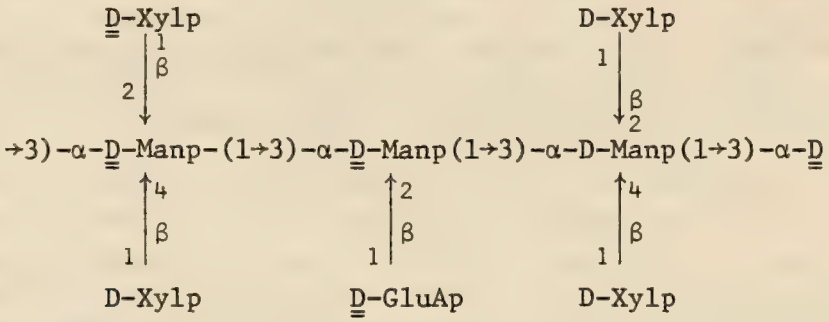
Serotype B and C isolates utilized creatinine much more readily than A and D, resulting in increased ammonia production and alkaline pH. Based on these findings, a creatinine-dextrose-brom thymol blue agar medium with pH 5.7 was developed as a single step culture method for separation of the two serotype groups. The isolates of A-D group produced no color change, while isolates of B-C group changed the medium from gold to blue within 24 to 48 hours. The medium can distinguish the two groups with 97 per cent accuracy by 48 hours.

2) On the Structure of the Capsular Polysaccharide from Cryptococcus neoformans Serotype C.

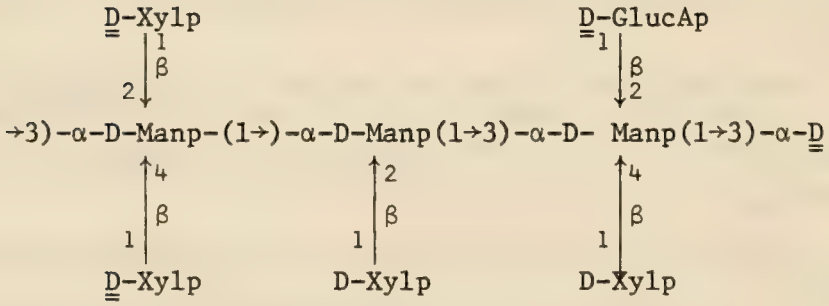
The cells of Cryptococcus neoformans are encapsulated with an antigenic polysaccharide which contains determinants defining their serotypes. Little has been known on the structures of the polysaccharide from each serotype. Chemical composition of a type A and a type B were studied by Rebers, et al. and Blandamer, et al., respectively but not the structure of the polysaccharide. Our study on the structure of the polysaccharide from serotype C showed the occurrence of a polysaccharide having an α1, 3 linked backbone of D-mannopyranoside residues every other one of which is doubly substituted with two terminal D-xylopyranosyl residues linked β1,2 and β1,4 respectively; and with the remainder of the mannosyl residues substituted with a terminal D-glucuronosidopyranosyl residues linked β1,2. Alternatively, the terminal D-glucuronic acid residue may be β1,2 linked to a mannosyl residue in the

neighboring mannosyl residue carries a single branch of β1,2 xyloyranoside (see below). Quantitative estimation of the component sugars in the carboxyl-reduced polysaccharide showed xylose, mannose and glucose in the ratio of 4:3:1.

1.



2.



Significance to Biomedical Research and the Program of the Institute:

1) Previous reports (1976, 1977) described the mycological, genetical and epidemiological differences between the serotypes A-D and B-C. The creatinine metabolism of C. neoformans described in the present report is an additional evidence that the species of C. neoformans contain at least two distinct species. On the basis of these findings, C. neoformans is now divided into two separate species: C. neoformans for the serotype A-D and C. bacillisporus for the serotype B-C.

2) The antigenic polysaccharide which contains determinants defining the serotype C of Cryptococcus bacillisporus revealed that the structure is very different from Cryptococcus neoformans serotype A.

Proposed Course:

Major effort will be directed to: 1) identify the biochemical lesions of the mutants (of C. neoformans) which lack the melanine pigment when grown on media containing phenolic compound; 2) analyze the genetic control of cychloheximide sensitivity.

Publications:

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2. Kwon-Chung, K.J., Bonder, R.H., Fethiere, A. and Blank, F.: Enigmatic relationship of two Microsporum species. Sabouraudia 15:325-332, 1977.

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8. Kwon-Chung, K.J.: Mycetoma caused by dermatophyte. First Int. Sym. Mycetoma, Benezuela, 1978. In press.

9. Young, N.A., Kwon-Chung, K.J., Kubota, T.T. and Jennings, A.E.: isseminated infection by Fusarium moniliforme during treatment for malignant lymphoma. J. Clin. Microbiol. In press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00058-05 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
The Pathogenesis and Chemotherapy of Herpesvirus Infections in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Dolin	Head, Medical Virology Section	LCI NIAID
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	V. Pons	Clinical Associate	LCI NIAID
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COOPERATING UNITS (if any)
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LAB/BRANCH
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NIAID, NIH, Bethesda, Md. 20014

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2	2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The pathogenesis of herpesvirus infections in man and in experimental animals is investigated by characterization of viral isolates and examination of humoral and cell-mediated immune responses. Rapid, convenient assays have been developed for the detection of Epstein-Barr virus in vitro, which are suitable for clinical use. Patients with infectious mononucleosis lack the ability to suppress outgrowth of EBV infected lymphocytes until convalescence. Systemic administration of adenine arabinoside continues to demonstrate efficacy in treatment of herpes simplex encephalitis, and trials in herpes zoster are nearing their conclusion. Acycloguanosine, a topically administered antiviral compound, is effective in prophylaxis of genital herpesvirus infections in guinea pigs.

Project Description:

Objectives:

1) To determine the pathogenesis, host defense mechanisms, and natural history of herpes virus infections in man, in both host-defense compromised and intact hosts.

2) To evaluate the efficacy of antiviral agents in the treatment of herpes virus infections in man. These infections include herpes simplex, herpes zoster, cytomegalovirus and EB virus.

3) To develop in vitro methods to study the activity mechanisms of actions, pharmacology, and interrelationships of antiviral agents.

4) To develop laboratory animal models in which to study pathogensis of herpes virus infections and the effect of antiviral substances.

Methods Employed:

1) Patients with infections with herpes simplex, herpes zoster, cytomegalovirus, and Epstein-Barr virus are referred to the Medical Virology Section from the Clinical Center, National Naval Medical Center, or other local hospitals or practicing physicians. Patients who are hospitalized are studied at the Clinical Center or at NNMC, while those who are ambulatory are seen in the outpatient clinic of the LCI.

2) Viruses isolated from the above patients are characterized in human diploid fibroblasts and in human fetal tracheal and intestinal organ cultures. The effect of antiviral compounds, such as adenine arabinoside, cytosine arabinoside, idoxuridine, phosphonoacetic acid and acycloguanosine on clinical isolates are studied in tissue and organ culture.

3) Host defense responses to herpetic viral infections are examined in the above patients. These include: (a) levels of circulating and locally produced antibodies; (b) cell-mediated immune responses; i.e., skin test sensitivity, mitogen and antigen stimulation of peripheral lymphocyte cultures, lymphocyte cytotoxicity against virus-infected target cells, and circulating B to T cell proportions; and (c) systemic and local (e.g., vesicle) interferon production. Similar studies are performed in animal model systems.

4) The clinical efficacy of ara-A in the treatment of herpes zoster and herpes simplex infections in the immunosuppressed is investigated in a randomized, controlled, double-blind study in collaboration with a NIAID-supported nationwide trial of which the principal investigator is project officer. Promising antiviral compounds, such as acycloguanosine are studied in animal models.

Major Findings:

1) Epstein-Barr Virus Infections

a. Establishment of assay system for detection of EBV: Because EBV can only be detected by immunofluorescence of transformed susceptible lymphocytes in vitro, a rapid convenient assay was developed in which susceptible lymphocytes are infected with a source of EBV, and ^{3}H thymidine incorporation is measured after an incubation of one week. This assay can employ fetal cord blood lymphocytes but can also be used with B-lymphocytes from an adult donor, regardless of the immune status of the donor. Stimulation indexes of >10 are generally found. This assay provides a rapid reproducible system in which virus can be quantitated and is suitable for detection of virus in a large number of clinical specimens.

b. Studies of cell-mediated immune function in patients with infectious mononucleosis: T lymphocytes from seropositive (antibody to EBV) individuals are able to suppress outgrowth of EBV infected autologous lymphocytes employing the previously described assay. In studies of 12 patients with infectious mononucleosis, T-cells obtained early during illness lacked this ability, while those obtained late had suppressed outgrowth. Lack of suppression was correlated with increased numbers of atypical lymphocytes which appeared to lack Fc receptors for IgG or IgM/(Haynes). The effects of suppression of outgrowth on EBV yield is being evaluated. These observations provide a mechanism for the control of lymphoproliferation observed during infectious monocucleosis.

2) Clinical trials of systemically administered ara-A in herpes simplex and herpes zoster infections in immunosuppressed patients.

a. Herpes Simplex Encephalitis: Because of the previous beneficial effects of ara-A upon mortality in herpes simplex encephalitis (NEJM 297:289, 1977), the placebo controlled trial was halted, and all patients enrolled in the trial received the drug in order to gather additional data on morbidity. Twenty-five additional biopsy proven patients (including four from this unit) were entered in the nationwide trial, with a mortality of 28%. Trials of more soluble analogs of ara-A, such as ara-AMP are planned for the future.

b. Herpes Zoster Varicella: An additional eleven patients were studied in the ongoing collaborative, placebo controlled studies. Data from these studies will be analyzed during the upcoming year. Examination of the experience at the NIH indicates that local x-irradiation and recent surgery are risk factors in the development of herpes zoster. Chemotherapy, including steroids was not itself associated with an increased risk in the Clinical Center population. There appeared to be a trend toward more frequent dissemination of herpes zoster in patients with splenectomies.

c. The outpatient clinic program for study of herpes genitalis is being discontinued because of the planned departure of the principal investigator.

3) Animal Model of Herpes Simplex Infections: Acycloguanosine, a potent inhibitor of HSV DNA polymerase, was evaluated in vitro and was found

to have an MIC of 0.1- to 1.0 µg/ml for HSV 1 and 2 in 30 strains. A 5% formulation in ointment was evaluated as topical therapy in HSV-2 intravaginal challenge of outbred guinea pigs in our previously described system. Administration of acycloguanosine 4x/day, 24 hrs. prior to challenge resulted in significant decreases in rates of infection, lesion scores, and virus shedding. No clinical toxicity was noted. Additional trials with administration of drug post challenge, as well as systemic administration, are currently underway. Acycloguanosine will undergo preliminary toxicology trials in humans during the upcoming year and may be of potential usefulness in treatment of human herpesvirus infections.

4) Characterization of Nucleic Acid From Varicella-Zoster Virus (VZV) Isolates: VZV isolated from localized and disseminated disease are grown in large pools in human diploid fibroblast cells (MA-196). Because of the high degree of cell association, quantities sufficient for biochemical analysis have been difficult to obtain. With the development of Freon extraction procedures (Mazur, Howley), sufficient quantities of VZV DNA were obtained to determine buoyant density (1.7006 g/cc in CsCl) and to begin analysis of fragments with restriction nuclease enzyme cleavage. Isolates from different clinical settings will be examined.

Significance to Biomedical Research and the Program of the Institute:

Herpes virus infections are a significant and widespread cause of morbidity in normal adults and children, as well as in neonates and in the immunosuppressed. Herpes simplex encephalitis is the most common fatal encephalitis in the United States. Herpes genitalis appears to be increasing in incidence in the United States and has been associated with cervical cancer. Varicella-zoster infections are more frequent and severe in immunosuppressed patients. The above studies suggest that ara-A may reduce mortality in herpes simplex ncephalitis and accelerate resolution of skin lesions in varicella-zoster infections. Definition of the precise clinical usefulness of this compound requires additional studies, and no other treatment of established efficacy is available. Studies on the pathogenesis and host response in herpes virus infections should further define their risk as well as suggest control measures. The availability of relatively nontoxic compounds such as ara-A offers a promising modality for the treatment of these infections. Studies with this and related agents can serve as models for the evaluation and use of other antiviral drugs which may be subsequently developed.

Proposed Course:

Clinical trials with antiviral agents will continue. The effect of ara-A on EBV in vivo in man will be studied by examining viral excretion, antibody response, and clinical parameters in immunosuppressed patients who receive the compound. The molecular mechanism of action of ara-A will also be investigated. Host response to herpesvirus infections in patients will continue to be studied, with continued emphasis on cell-mediated immunity and its relationship to latency and reactivation. Antigens which elicit cell-mediated immune responses will be characterized. Varicella-zoster isolates

from a variety of clinical settings will be analyzed for nucleic acid content by restrictive nuclease fragments to detect potential clinical correlates. Patterns of excretions of EBV in immunosuppressed patients will be examined, in a prospective study, to define potentially associated clinical illness.

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1. Reichman, R.C., Dolin, R., Vincent, M. and Fauci, A.S.: Cell-mediated cytotoxicity in recurrent herpes simplex infections in man. Proc. Soc. Exp. Biol. Med. 155:571-576, 1977.

2. Whitley, R.J., Soong, S.J., Dolin, R., Galasso, F.J., Ch'ien, L.T., Alford, C.A. and the NIAID Collaborative Study Group: Adenine-arabinoside therapy of biopsy proven herpes simplex encephalitis. N. Engl. J. Med. 297:289-294, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 A1 00154-03 LCI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunologic, Neurophysiologic, Biochemical and Cellular Events in Immediate Hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	G. Raphael	Guest Worker	LCI	NIAID
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COOPERATING UNITS (if any)

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LAB/BRANCH

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4 8/12	3 8/12	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The immunologic release of the mediators of anaphylaxis from IgE-sensitized human lung tissue is accompanied by increases in cyclic AMP and cyclic GMP; responses attributable to histamine interacting with H-2 and H-1 receptors, respectively. Prostaglandin release is also partly due to histamine stimulation. Mucus glycoprotein secretion from submucous glands and goblet cells is stimulated by mecholyl, histamine and anaphylaxis. Rat mast cells contain both peroxidase and superoxide dismutase in their granules. These granule-associated-enzymes are not released like histamine and provide an additional source of mediators. Histamine stimulation of rat mast cells fails to cause an increase in mast cell cyclic nucleotides or influence degranulation. Human platelets contain arylsulfatase type IIA and IIB in their secretory granules. Both platelet arylsulfatase and mast cell peroxidase are capable of destroying SRS-A. Human asthmatic subjects have excessive sensitivity to alpha adrenergic and cholinergic stimulation and deminished beta adrenergic responses in comparison with normal controls. Subjects with allergic rhinitis have normal alpha adrenergic responsiveness but the same degree of cholinergic and beta adrenergic dysfunction as the asthmaties.

Project Description:

Objectives:

The goals of my laboratory are to expand our knowledge of all aspects of immediate hypersensitivity reactions. Our particular expertise and focus is on defining the biochemical events which accompany and control the triggering of allergic reactions, the neurophysiologic mechanisms which potentially modulate these events and the characterization of the mediators of anaphylaxis.

We are concentrating on four primary areas: 1) Employing human lung tissue, we study "asthma in a test tube" in order to understand the immunologic triggers, biochemical concomitants and neurophysiologic controls of mediator release as well as a search for new effector molecules; 2) Rat peritoneal mast cells are studied as a pure source of effector cells which may be readily studied in vitro and may amplify as well as initiate new areas of interest in relationship to the human lung model; 3) Clinical allergic asthma is being evaluated in an extensive protocol designed to expand our understanding of the neurophysiologic mileau of allergic individuals; and 4) Studies involving other cell types involved in immediate hypersensitivity such as the eosinophilic leukocyte, the platelet and the mucous cell are being continued in order to define their role in human disease.

Methods Employed:

1. Human lung model: Lungs removed, usually for cancer resection, almost always contain large amounts of normal, functioning lung tissue. We obtain these lungs in cooperation with the surgical and pathology departments of all the major hospitals in the Washington and Maryland areas. The lung tissue is washed, fragmented and replicated into 200 mg "chunks" and subsequently incubated in dilutions of allergic serum for 2 hours at 37°C or 18 hours at 25°C. After this "sensitization" period, the serum is removed and the fragments challenged with allergin. The interaction of the allergin with tissue (mast cell) bound IgE induces the release of histamine, slow reacting substance of anaphylaxis (SRS-A), eosinophil chemotactic factor of anaphylaxis (ECF-A), and prostaglandin E_1 and $F_{2\alpha}$ (PGE_1 and $PGF_{2\alpha}$). These mediators may be assayed on the isolated terminal portion of the guinea pig's ileum (Histamine, SRS-A), chemotactic chambers (ECF-A) or by radioimmunoassay (PGE_1, $PGF_{2\alpha}$).

We have been determining alterations in the tissue levels of cyclic AMP and cyclic GMP after treating lung with various agonists in order to define the role these nucleotides play in modulating the release of mediators. Cyclic AMP may be measured by either a protein kinase binding assay or radioimmunoassay while cyclic GMP is determined by radioimmunoassay after acetylation or succinylation.

Mucus glycoproteins are monitored by incorporating ^{3}H-glucosamine into the mucous glands during an 18 hour incubation period. Secretions are dialyzed against 6M urea, applied to sepharose 2B columns, and analyzed by polyacrylimide gel electrophoresis and ion exchange chromatography.

2) Rat mast cells: Rat peritoneal and pleural mast cells are obtained from freshly sacrificed male Sprague-Dawley rats by lavage of these cavities. The peritoneal cells include 5% mast cells (or approximately 500,000 to 1 million/animal). The cells are used either in mixed cell suspensions or after purification by centrifuging them into albumen cushions. Peroxidase is measured spectrophotometrically using O-tolidine as substrate and histochemically with benzadine dihydrochloride.

Mast cells granules are isolated with intact perigranular membranes by sonication and centrifugation through sucrose cushions. Granules free of membranes are obtained by osmotic lysis of mast cells.

3) Clinical asthma study: The methods employed in this study are covered in detail in the clinical protocol and include: a) measurement of cutaneous blood flow by ^{133}xenon disappearance; b) pupillary responses as measured by a binocular pupillometer; c) airway obstruction as measured by flow-volume curves with and without helium inhalation; d) serum cyclic nucleotide responses after beta adrenergic stimulation; and e) eccrine sweat responses as measured after intracutaneous Mecholyl administration.

4) Platelets: Platelet rich plasma and purified platelets are employed for the isolation of arylsulfatases. SRS-A is obtained by treating rats intraperitonally with anti-rat IgE and subsequently lavaging the peritoneal cavity. Both the platelet arylsulfatase and rat SRS-A are partially purified by chromatography (DEAE + G-200 for arylsulfatase and alcohol extraction followed by base hydrolysis and amberlite XAD-7 for SRS-A).

Major Findings:

1) Analysis of the cyclic nucleotide content of lung tissue after anaphylaxis revealed a massive and rapid increase in the cyclic AMP and cyclic GMP contents. The pattern of nucleotide formation paralleled mediator release: both histamine and prostaglandins were assayable at the time of nucleotide response. While aspirin pretreatment did not prevent these responses (suggesting that they were not related to prostaglandins), antihistamines did affect the phenomena. H-1 antagonists (pyrilamine and diphenhydramine) prevented the cyclic GMP increase while H-2 antagonists (cimetidine or metiamide) prevented the cyclic AMP elevation.

Histamine was also found to cause increases in lung cyclic AMP and cyclic GMP. Similar to the findings after anaphylaxis, histamine-induced increases in cyclic AMP were prevented by H-2 antagonists while the cyclic GMP elevations were prevented by H-1 receptor antagonists.

The prostaglandin formation accompanying anaphylaxis was found to be primarily $PGF_{2\alpha}$, to be partially prevented by H-1 receptor antagonists, and to be reproduced by both histamine and selective H-1 receptor agonists. These data indicate that histamine is responsible for 50% of the $PGF_{2\alpha}$ synthesis accompanying anaphylaxis.

Pretreatment of lung with antihistamine failed to influence the quantity of histamine and SRS-A released during anaphylaxis. The capacity of histamine to modulate the release of mediators from mast cells was examined. It was found that histamine failed to influence the release reaction despite being able to increase the cyclic nucleotide levels of lung tissue.

Histamine-induced prostaglandin formation by lung was further analyzed. $PGF_{2\alpha}$ formation was found to occur from peripheral lung cells while PGE_2 was synthesized by bronchial muscle cells. The capacity of KCl and carbachol to reproduce histamine-induced PGE_2 formation from bronchial smooth muscle cells but not $PGF_{2\alpha}$ from peripheral tissue indicates that PGE_2 arises from contracting muscles while $PGF_{2\alpha}$ is independent of muscular action.

2) A procedure for the isolation of rat mast cell granules with intact perigranular membranes was developed. Employing purified mast cells, the technique involves sonication followed by centrifugation through sucrose. This granule preparation closely reflect intracellular granules and permits a close analysis of granule constituents.

The enzyme peroxidase was localized to mast cell granules both histochemically and enzymatically. Although peroxidase and SOD were localized to the mast cell granule, neither of these enzymes was relased during degranulation in contrast to histamine. It was found that both peroxidase and SOD were eluted from mast cell granules by hypertonic washes and that exposing degranulated mast cells to hypertonic solutions also resulted in peroxidase release. Therefore, the constituents of the granule matrix, such as SOD and peroxidase, represent an additional source of mediators generated during anaphylactic reactions. Mast cell granular peroxidase was capable of destroying SRS-A in the presence of H_2O_2.

Mast cell granules injected into the skin of rats and monkeys were found to cause significant inflammatory reactions. Granules isolated with intact perigranular membranes caused a polymorphonuclear rich infiltrate apparent in 2 hours which peaked by 8 hours. Subsequently, an intense mononuclear infiltrate replaced the PMN's and peaked at 24-48 hours. Granules isolated free of membranes and washed free of elutable mediators injected into skin caused a different reaction. No 2-8 hour infiltrate was observed. Instead, starting at 8 hours and peaking at 24-48 hours, an intense mononuclear infiltrate occurred. These data indicate that the mast cell granule is capable of causing an inflammatory reaction in 2 phases; an earlier acute reaction related to elutable mediators and a later, chronic-type inflammation caused by the granular constituents.

3) Human platelets were found to contain arylsulfatase which were characterized by size, charge, elution pattern from DEAE cellulose column chromatography, susceptability to inhibition by sulfate and phosphate and classified as types IIa and IIb. Partially purified A-S was capable of degrading rat SRS-A suggesting a possible role of the platelet in destroying SRS-A generated by allergic reactions.

4) Human lung tissue was found to incorporate ^{3}H-glucosamine into mucus glycoprotein by the following criteria: 80% of incorporated glucosamine had a molecular size of greater than 200,000, a carbohydrate to protein ratio of 70:30, and similar behavior with incorporated $^{35}SO_4$, or ^{3}H-threonine. Gel filtration of ^{3}H-labeled mucus revealed 2 peaks of activity - one of greater than 2,000,000 molecular weight and the other of 200,000. These peaks could further be separated by electrophoresis in polyacrylamide gels. Secretion of these ^{3}H-labeled macromolecules was induced by mecholyl, histamine, several prostaglandins and anaphylaxis.

5) The autonomic responses of asthmatic subjects have been compared with those of subjects with allergic rhinitis and normal controls. Alpha adrenergic function was assessed by pupillometry and cutaneous vascular responses. In both instances, the subjects with asthma were significantly more sensitive than both allergic rhinitis and normal control subjects. Beta adrenergic function has been analyzed by the cardiovascular and cyclic AMP responses to intravenous isoproterenol in these same subjects. Patients with either asthma or allergic rhinitis have significant impairment of both beta adrenergic responses as compared with controls. Parasympathetic function has been analyzed by pupillometry, sweat responses and bronchial challenge. Again, patients with either allergic rhinitis or asthma demonstrate the same degree of exagerated responsiveness in comparison with normal controls. Therefore, asthmatics are selectively more responsive to alpha adrenergic stimulation while both asthmatics and subjects with allergic rhinitis have increased cholinergic and deminished beta adrenergic responses.

Significance to Biomedical Research and the Program of the Institute

Increased understanding of the mechanisms of immediate hypersensitivity and its controls may eventually make it possible to better treat the 44 million Americans with allergic rhinitis, the 9 million with asthma and the 22 million with urticaria. The model systems employed permit sophisticated analysis of many of the aspects involved in vivo. As a direct consequences of our previous studies, a much finer appreciation of the pharmacology of anti-allergic drugs has been developed.

Appreciation of the potent effects of mediators such as histamine upon lung cells other than those generally envisioned as target tissues in asthma (smooth muscle, vascular bed, mucous gland) underlines our need for further study of the consequences of anaphylaxis. The responses of lung cells to increases in cyclic AMP and cyclic GMP are unknown at present, but it is anticipated that multiple effects are produced. Among the effects are the synthesis of various prostaglandins which are themselves potent stimuli. As antihistamines prevent these responses, it is evident that antihistamine therapy in asthma warrants a re-examination. Further, the absence of modulation of the immunologic release of mediators by both histamine and antihistamines indicates that the increasingly popular use of H-2 antagonists for peptic ulcer disease should have no adverse influence upon allergic individuals.

Development of procedures by which mast cell granules may be isolated in the same state which they are maintained intracellularly permits a rather sophisticated analysis of their biologic properties. We have thus far localized 2 potent enzymes to the granular matrix and have suggested that the matrix represents a hitherto unrecognized source of additional mediators of anaphylaxis. This observation has preliminarily been followed up by the idenification of the histologic responses initiated by the injection of granular matrix into rat and monkey skin. The responses which have been uncovered probably explain the delayed skin test and bronchial provocation response which occasionally follow antigen challenge by 8-24 hours. It seems likely that allergic individuals are variably responsive to mast cell granular proteins and that the most responsive subjects will develop these delayed responses. It also seems likely that the potent enzymes in the mast cell granule matrix account for several unexplained pathologic and clinical consequences of allergic reactions: the desquamation of the bronchial mucosa and the thickening of the basement membrane in asthma and the inflammed, recalcitrant nasal congestion seen in a portion of subjects with perennial allergic rhinitis. Further, the capacity of mast cell granule peroxidase and human platelet arylsulfatase to destroy SRS-A suggests that both these cells may contribute to allergic reactions by eliminating this mediator.

The study of human lung mucus synthesis and secretion is still in its preliminary stages but we can state that the macromolecules which constitute mucus glycoproteins appear in 2 molecular sizes and that certain hormones and mediators generated during lung anaphylaxis are capable of stimulating secretion. Analysis of mucus secretion should provide insights into both the pathogenesis and treatment of bronchorrheic states.

The definition of the complex autonomic abnormalities inherent in subjects with allergic asthma provides the initial step in classifying asthmatics by these physiologic responses. The identification of significant alpha adrenergic sensitivity suggests that a careful consideration of alpha adrenergic blocking agents in the treatment of asthma is indicated and that the use of pharmacologic agents which include alpha adrenergic agonists in asthmatics should be undertaken with caution. The observation that subjects with allergic rhinitis share the same dysfunction of beta adrenergic and cholinergic responsiveness as do asthmatics suggests that these abnormalities may not be sufficient to lead to asthma.

Proposed Course:

The study of human lung anaphylaxis is being extended to: a) Examine the effects of interferon. b) Analysis of thromboxane B_2 release. c) Analysis of the factors responsible for protaglandin generation and d) Identification of the IgE bearing cells in lung, the histamine containing cells and the heparin containing cells in order to determine if only the mast cell participates in allergic reactions.

Rat mast cells will be studied for: a) Responses to histamine; b) presence of histamine receptors and c) calcium fluxes.

Rat mast cell granules will be further analyzed for: a) Smooth muscle contracting activity; b) kallikrein activity; c) complement activation; and d) definition of the factors responsible for: a) mononuclear cell chemotaxis and b) phagocytosis.

Asthmatic subjects with: a) exercise-induced asthma, b) intrinsic asthma and c) more severe allergic asthma will be added to the present study. An additional protocol for analysis of histamine sensitivity in asthma is being initiated.

In addition, the Allergic Diseases Section is initiating an analysis of food hypersensitivity. The first studies will deal with identification of cells involved in the allergic responses of animal and human gastrointestinal tracts and identification of subjects with well-defined food allergy.

Publications:

1. Kaliner, M.: Human lung tissue and anaphylaxis. The role of cyclic GMP as a modulator of the immunologically-induced secretory process. J. Allergy Clin. Immunol. 60: 204-211, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 A1 00155-03 LCI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Phagocytic Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	John Davis	Guest Worker	LCI	NIAID
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SECTION
Bacterial Disease Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3 1/12	3 1/12	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanism of leukocyte activation by chemotactic factors has been studied using electrophysiology, fluorescent probe, surface charge and ultrastructural techniques.

Studies assessing the mechanism of modulating leukocyte locomotion indicate that limited secretion of specific granules, which accompanies chemotaxis, is associated with increased cell adhesiveness and increased availability of chemoattractant receptors. Vigorous exocytosis is associated with depressed chemotaxis, decreased availability of chemoattractant receptors, chemoattractant hydrolysis by secreted products and markedly increased cell adherence and aggregation. Human pyrogen has been shown to be a potent stimulator of neutrophil exocytosis.

Studies of the two populations of neutrophils we had identified previously indicate that during the neutropenia that follows in vivo endotoxin or hemodialysis, a subpopulation of neutrophils with poorly demonstrable Fc receptors is the predominant neutrophil left in the circulation. Clinical studies assessing the effect of pharmacologic agents on the neutrophil subpopulations in normal subjects, patients with recurrent infection and host defense defects are underway.

Project Description

Objectives:

1) Study the mechanism of leukocyte chemotaxis.

2) Study the phenomenon of deactivation of leukocyte chemotaxis and to relate this to the pathophysiology of leukocyte dysfunction syndromes.

3) Study neutrophil subpopulations in normal subjects and in patients with neutrophil dysfunction.

4) Study the mechanism of neutrophil margination and aggregation at capillary beds prior to cell migration into tissues.

5) Study the mechanism of mobilization and secretion of neutrophil lysosomal granules, study the relationship of the secretory process to neutrophil function and dysfunction and to study how neutrophil secretory products may influence homeostasis.

6) Characterize and define the mechanism of abnormal neutrophil chemotaxis following thermal injury.

7) Study the role of fibroblast secretory products in the inflammatory response.

8) Study the effects of pharmacologic agents on leukocyte function *in vitro* and *in vivo*.

9) Assess the chemotactic activity of histamine for eosinophils *in vivo*.

Methods Employed

Peripheral blood leukocytes were separated from heparinized whole blood by dextran sedimentation and Hypaque-Ficoll separation. Chemotaxis was evaluated using a radioassay employing ^{51}Cr labeled leukocytes and a double micropore filter system or a single filter measuring the distance migrated into the filter by the population of responding cells. Cell adherence was measured by quantitating the number of leukocytes adhering to plastic surfaces or to nylon wool columns. Phagocytosis, bactericidal capacity, nitroblue tetrazoliun dye reduction, hexose monophosphate shunt activity and superoxide generation were measured using standard techniques. PMN receptors for the chemoattractant f-met-leu-phe were quantitated as published previously.

The electrophysiology of cultivated human macrophages was studied using standard intracellular recording techniques. The fluorescent probe dipentyloxacarbocyanine was used to measure potential changes in neutrophils. Leukocyte surface charge was measured with a Zeiss cytopherometer. Intracellular calcium was localized in human neutrophils by the intracellular precipitation of

pyroantimonate anion followed by microprobe analysis and studies with the metal chelators (EDTA and EGTA).

The cytoskeleton of polymorphonuclear leukocytes was studied during conditions of chemotaxis and chemokinesis using 0.45 μm micropore filters. These small filters impede leukocyte migration but permit pseudopod penetration and a fixed orientation of leukocytes is thereby established. Orientation of living cells was monitored by well established techniques. Secretion of leukocyte granule enzymes were monitored by standard spectrophotometric assays. Polymorphonuclear leukocyte granules were separated and fractionated using sucrose-gradient techniques and various enzyme markers were utilized to identify granule types.

Neutrophils were fractionated into subpopulations based on their ability to rosette IgG coated erythrocytes. In some studies, in normal human volunteers, the effects of intravenous endotoxin on neutrophil subpopulations was examined.

Major Findings

1. During chemotaxis neutrophils selectively secrete their specific granules. It has been shown that this secretory event modulates chemotactic responsiveness. With limited exocytosis there is increased chemoattractant binding to the cell membrane. This appears related to translocation of granule membrane, which contains the chemoattractant receptors, to the cytoplasmic membrane. This may provide the mechanism for membrane turnover during sustained locomotion. Following vigorous exocytosis both chemotaxis and cell orientation in a gradient of chemoattractant are inhibited. This inhibition is related to decreased binding of a chemotactic factor to the cell. The latter has been associated with decreased peptide affinity and increased hydrolysis of chemoattractant by granule products secreted extracellularly.

2. Exocytosis of neutrophil granules *in vitro* increases neutrophil adhesiveness and aggregation. This has been related to neutralization of the net negative surface charge of the cell membrane and facilitation of cell-cell and cell-surface adhesion. In addition, submembranous deposition of cations (calcium) at the leading edge of cells in a gradient of chemoattractant was observed and speculated to facilitate fusion of intracellular granules with the cytoplasmic membrane. In this regard, it is of interest that the specific granules, which are most accessible to extracellular release, are more negatively charged than the azurophil granules.

3. Highly purified leukocyte pyrogen causes selective secretion of human neutrophil specific granules *in vitro* and rabbit neutrophils *in vivo*. In addition, pyrogen stimulates neutrophil nitroblue tetrazolium dye reduction, hexosemonophosphate shunt activation and superoxide generation.

4. Following intravenous injection of endotoxin into normal human subjects there is decreased lysozyme content and chemotactic responsiveness of the circulating neutrophils.

5. Twenty minutes following intravenous administration of E. coli endotoxin to normal volunteers, during the period of neutropenia, the predominant circulating neutrophil is a "subpopulation" without readily demonstrable IgG (Fc) receptors. Plasma obtained at the time of neutropenia increases neutrophil adhesiveness. In related studies, twenty minutes after initiating hemodialysis in patients with chronic Schizophrenia, also during a period of neutropenia, the remaining circulating neutrophils are significantly enriched with a subpopulation of neutrophils with poorly demonstrable Fc receptors. Plasma obtained at this time during hemodialysis increased the adhesiveness and aggregation of control leukocytes.

6. Hydrocortisone sodium succinate reversibly inhibits adherence of IgG sensitized erythrocytes to human peripheral blood neutrophil monolayers suggesting hydrocortisone interferes with the availability of the neutrophil Fc receptor for binding.

7. Administration of prednisone to normal human subjects inhibits in vitro neutrophil and eosinophil adherence to nylon wool. In vivo prednisone also inhibits eosinophil but not neutrophil chemotaxis.

8. Collection of human neutrophils by filtration leukapheresis for subsequent intravenous administration results in functional changes of the neutrophils attributable to degranulation and secretion of granule contents. Colchicine pretreatment of filtration leukapheresis donors significantly reduces these adherence induced changes.

9. Human fibroblasts cultured in vitro secrete at least two chemoattractants which, based on their elution from G-75 Sephadex chromatography columns, have molecular weights of greater than 150,000 and less than 5,000 daltons. These attractants appear distinct from collagen and other previously described chemotactic factors. They are protein in nature and attract both polymorphonuclear leukocytes and monocytes. Elaboration of this material in vitro is inhibited by colchicine (10^{-6} M) and hydrocortisone sodium succinate (1 mM).

10. In studies of patients with recurrent pyrogenic infections, a patient with abnormal neutrophil chemotaxis whose neutrophils are missing a membrane glycoprotein has been identified.

Significance to Biomedical Research and the Program of the Institute

The accumulation of leukocytes at inflammatory, immune and allergic sites is critical for appropriate responses. Understanding the physiologic basis for events regulating these processes will provide the basis for therapeutic manipulation.

One of the first steps in leukocyte mobilization from the blood stream is increased cell adhesiveness to the endothelium. This is followed by local leukocyte aggregation and then diapedesis. Our finding that limited degranulation and exocytosis of intracellular granules markedly enhances these events, together with the observation that human pyrogen initiates these processes, provides a clue as to what controls margination and then emigration of leukocytes

from the blood stream. The related observation that vigorous degranulation in vitro inhibits chemotaxis suggested that some acquired chemotactic defects relate to excessive degranulation in vivo. In support of this is our finding that following thermal injury the severity of the acquired chemotactic defect is linearly related to the amount of intracellular lysozyme released. Clinically, it is of interest that the degranulation and defective chemotaxis precedes the severe and often lethal pyrogenic infections that follow thermal injury.

In related studies we confirmed that neutrophil specific granules contain lactoferrin and showed this is secreted when cells are incubated with pyrogen. Lactoferrin irreversibly binds iron which is then sequestered in the reticulo-endothelial system. Another specific granule component, B_{12} binding protein, may effect B_{12} related events when secreted from the neutrophil. Thus the potential regulatory role of neutrophil products on a number of systems is interesting and currently under investigation.

Two observations from our studies of the mechanism of the phenomena of degranulation, cell adhesiveness and chemotaxis may be particularly important to the cell biology of chemotaxis and perhaps relevant to clinical studies as well. We have shown that leukocyte adherence, aggregation and secretion are under the modulating influence of electrostatic forces. Development of techniques for controlling these forces in vivo may have potential clinical use. Our data that neutrophil specific granules are a potential source of new cytoplasmic membrane and chemoattractant receptors is intriguing in terms of understanding the basis for membrane turnover during chemotaxis. These studies need to be extended and explored further.

The studies of the effect of steroids on PMNs are interesting and have shown that prednisone in vivo inhibits both neutrophil and eosinophil adherence and eosinophil chemotaxis. Hydrocortisone sodium succinate also blocks the expressability of neutrophil Fc receptors and inhibits chemotactic factor induced changes of neutrophil and macrophage membrane potential. These data contribute to our understanding of the physiologic basis for steroid action on these cells.

The use of fluorescent carbocyanine dyes to measure leukocyte potential is emmerging as a simple and rapid test of cell responsiveness. We are currently evaluating this test in normal subjects and patients with neutrophil dysfunction to determine the usefullness of using this as a routine test of leukocyte function. Other fluorescent probes, designed to monitor membrane fluidity and viscosity, are also being utilized in this system.

The continuation of our studies of neutrophil subpopulations has indicated that one population of cells (PMNs with readily demonstrable Fc receptors) is particularly available for margination. We are presently extending these studies of neutrophil subpopulations to explore their kinetics of circulation, and their functional role. In addition, the effect of various disease states and pharmacologic agents on these two neutrophil populations in man and in animals is under study.

Proposed Course

We plan to continue some of the studies outlined above.

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I N D E X

LABORATORY OF INFECTIOUS DISEASES

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SUMMARY STATEMENT - Annual Report of the

Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
October 1, 1977 to September 30, 1978

The main focus of the research programs of LID continues to be the natural history and prevention of the three major uncontrolled, acute infectious diseases of man--acute respiratory tract disease, viral hepatitis and acute gastroenteritis. Broad advances were made in each of these areas during 1977-1978.

HEPATITIS

Hepatitis B - Because of its potential usefulness as an immunogen for prevention of hepatitis B, the surface antigen of hepatitis B virus (HB_sAg) has been studied intensively. Two pilot lots of HB_s Ag vaccine, one subtype adw and the other subtype ayw, were recently prepared by Dr. J. Gerin of the MAN Laboratory (NIAID contract #82572) in a collaborative program. Following completion of extensive safety tests in chimpanzees, clinical trials were initiated in adult volunteers to evaluate safety and antigenicity. This phase of the initial human trials of the monovalent vaccines is currently in progress and the results to date are encouraging. A bivalent vaccine, containing the two monovalent components has passed its safety and antigenicity tests in chimpanzees and will be evaluated in volunteers in late 1978. Following completion of this evaluation it is anticipated that human efficacy trials will begin in March, 1979. Epidemiologic data is currently being collected to serve as a baseline for the efficacy trials.

Although a hepatitis B vaccine prepared from HB_sAg purified from the plasma of chronic carriers of the antigen is feasible and probably cost-effective for its contemplated uses in the United States, such a vaccine probably will not be cost-effective for the people most in need of a vaccine in the developing world. Therefore, there is a need for additional studies on alternative methods of purification and inactivation, comparisons of monovalent vs. bivalent or polyvalent vaccines, studies of different combining ratios of mononvalent vaccines into bivalent preparations, different vaccine dosages, different vaccination schedules, different routes of administration, as well as evaluation of possible adjuvants and their interaction with vaccine preparations and further characterization of the immunizing antigens themselves.

An observation made during evaluation of vaccine in chimpanzees suggested that type B hepatitis could be prevented by vaccination after exposure. Although the data were limited there was evidence that such post-exposure vaccination might be more effective than administration of hepatitis B immune globulin following exposure. Additional studies in chimpanzees are planned to confirm and extend this important observation.

Because hepatitis B virus (HBV) occurs in a titer as high as 10^8 infectious particles/ml in the plasma of certain chronically infected individuals, it is possible to purify infectious virus by large volume ultracentrifugation and to characterize the resulting material. Recent collaborative studies carried out with Dr. Gerin revealed that hepatitis B virions are heterogeneous; three populations of particles were separated by isopynic banding in cesium chloride. The lowest density population lacks DNA polymerase activity and probably contains little if any nucleic acid. The highest density population is strongly positive for polymerase activity and contains double-stranded circular DNA that can be extracted and characterized. Most interesting is the intermediate density population which is heterogeneous, weakly polymerase positive and which contains nucleic acid with a molecular weight 80-90% of that of the highest density particles. Thus, these intermediate density particles have all of the biophysical characteristics of defective interfering particles, entities that in other virus infections have been shown to inhibit the synthesis of fully infectious virus in vitro and to modulate virus infection in vivo in certain laboratory animal model systems, converting a rapidly progressive infection to a persistent or chronic one. However, to date such a defective interfering particle system has not been detected in a human viral disease. Nonetheless, the existence of intermediate density virus particles may have important implications for the isolation of HBV in vitro. Our failure to grow HBV in vitro may result from the fact that the virus is always found in the presence of a vast excess (up to a million-fold more particles) of viral coat material (HB_sAg) as well as a variable concentration of defective interfering particles. The former can compete for receptor sites on sensitive cells, and the latter can inhibit the synthesis of infectious virus during coinfection of cells. To examine this possibility, preparations of high density and intermediate density hepatitis B virions are being purified by isopynic and rate zonal centrifugal procedures under appropriate containment in Dr. Gerin's laboratory. These preparations are being titered for infectivity in chimpanzees and will be used for attempts to isolate hepatitis B virus in a number of tissue culture cell lines. (Purcell, McAuliffe, Daemer)

Hepatitis A - As with hepatitis B virus, hepatitis A virus (HAV) has resisted all attempts at cultivation in vitro. The reason for this is not understood, but it may be related to the observation that hepatitis A virus particles with different densities in cesium chloride occur naturally in infected tissues and in gastrointestinal tract contents. Whether type A virus infection produces a large number of defective-interfering particles or whether the different densities are the result of cleavage of capsid proteins, as occurs with parvoviruses and picornaviruses, remains to be determined. Large scale purification procedures recently developed by Dr. Moritsugu when he was a Visiting Scientist in LID may help to answer these questions. The recent development of an immunofluorescence technique for hepatitis A virus antigen (HA Ag) has provided another powerful tool for evaluating attempts to isolate virus in vitro. That hepatitis A antigen (HA Ag) can be detected in cultured cells was very recently shown in a collaborative study with workers in the Bureau of Biologics, FDA; marmoset hepatocytes obtained during acute HAV infection

were cultured in vitro, and specific HA Ag was detected in the cytoplasm of the cells through their first two passages in vitro. By third passage the hepatocytes no longer contained detectable HA Ag. Although these cells, like hepatocytes from patients with type B hepatitis, lost their viral antigen upon repeated subculture, they provide an opportunity to study other approaches to isolation, including cocultivation, induction with anti-metabolites, use of helper viruses, etc. (Moritsugu, Matheisen, Feinstone, Purcell, Shimizu)

Non-A, Non-B Hepatitis - A series of unsuccessful attempts to transmit non-A non-B hepatitis virus(es) to primates were carried out in past years. However, recently two successful transmission studies were completed simultaneously, one by workers in the Bureau of Biologics and the other by workers in the Clinical Center Blood Bank in collaboration with our laboratory. In both studies the incubation period in chimpanzees was comparable to that for non-A, non-B hepatitis in man, and both biochemical and histologic evidence of hepatitis was obtained. In our collaborative study, plasma or serum from patients with chronic non-A non-B hepatitis as well as from patients with acute infections transmitted disease to chimpanzees. Thus, proof of chronic carriage of non-A non-B viral hepatitis agents has been obtained. In a collaborative confirmatory study, serial plasmapheresis units and serial liver biopsies were obtained from experimentally infected chimpanzees. Fluorescein-labeled convalescent serum from the original patients with non-A, non-B hepatitis and from experimentally infected chimpanzees have been prepared and are being tested against acute phase, frozen liver biopsies in an attempt to detect a viral antigen. In addition, the plasmapheresis units will be subjected to ultracentrifugal separations and studied by radioimmunoassay and immune electron microscopy techniques in a search for viral antigens. Also, previously infected chimpanzees are being cross-challenged and pools of infectious plasma are being identified for use in subsequent infectivity titrations. It is likely that application of the many immunologic and biophysical techniques developed for the characterization of hepatitis A and hepatitis B viruses will ultimately lead to the identification and characterization of the non-A non-B hepatitis viruses. (Feinstone, Purcell)

RESPIRATORY SYNCYTIAL VIRUS

Genetics - RS virus is the major etiologic agent of bronchiolitis and pneumonia of early life and the need for effective immunoprophylaxis has been clearly established in studies performed throughout the world. This virus has a non-segmented genome and does not undergo genetic reassortment. Based on the probable molecular weight of the RS virus genome, the existance of at least 6-7 complementation groups is likely. In this laboratory ts mutants were grouped previously into 3 complementation groups based on their ability to complement one another at the restrictive temperature of 38° or 39°C. Six ts mutants of RS virus isolated in the laboratory of Dr. Craig Pringle (Glasgow, Scotland) were tested in our laboratory by complementation using the prototypes from the NIH and Glascow complementation groups. On the basis of this analysis, we were tentatively able to identify 2 new complementation groups of RS virus.

Three of Pringle's ts mutants can be grouped into our Group A, one mutant in a new Group D and two mutants in a new Group E.

In terms of future directions it is now clear that the ts-2 mutant, a member of complementation group B, is the most attenuated of the mutants thus far tested. This mutant, that is defective in the early function of cell penetration, has reduced infectivity for the chimpanzee and is completely attenuated for this host. The gene that is the locus of the ts-2 mutation offers a desirable target for further attempts at mutagenesis if the ts-2 mutant does not prove to be acceptable as a vaccine strain for infants and young children.

Last year 5 ts mutants of RS virus were evaluated for virulence in seronegative chimpanzees. Two of the mutants, ts-1 and ts-7, induced rhinitis similar to that seen during infection with wild type virus. The more defective derivatives of ts-1 into which additional mutations were introduced, i.e., ts-1 NG-1 and ts-1 NG-16, induced very mild rhinitis which was less extensive than that observed during infection with wild type virus, ts-1 or ts-7. The ts-2 mutant was less infectious for chimpanzees than wild type virus or the other mutants. Significantly, the ts-2 mutant did not induce any signs of respiratory tract disease.

Viruses shed by chimpanzees infected with the various ts mutants of RS virus were analyzed for ts phenotype by assaying efficiency of plaque formation at permissive and restrictive temperatures. None of the 5 mutants tested (ts-1, ts-1 NG-1, ts-1 NG-16, ts-2 and ts-7) was completely stable genetically during replication in chimpanzees. In most instances the genetically altered virus was less temperature sensitive than the parental ts mutant, but was demonstrably more temperature sensitive than wild type virus. The genetically altered viruses produced by chimpanzees infected with either derivative of ts-1, i.e., ts-1 NG-1 or ts-1 NG-16, retained a degree of temperature sensitivity with the exception of one isolate of ts-1 NG-16. Of particular interest was the finding that only ts^+ ("wild type-like") virus was shed by two of the five chimpanzees infected with the ts-2 mutant. Two clones of "revertant" ts-2 (i.e., virus with the wild type ts^+ phenotype) were evaluated in chimpanzees to determine whether virulence had been restored. Chimpanzees shed these viruses in as high a titer as wild type RS virus. Significantly, however, the chimpanzees infected with "revertant" ts-2 failed to develop any signs of upper respiratory tract disease. This observation was confirmed when 4 other clones of "revertant" ts-2 were evaluated in owl monkeys. None of the owl monkeys developed rhinorrhea although each animal became infected. These primates shed considerably less virus than monkeys infected with wild type virus. The observations made during these studies stress the importance of evaluating candidate live vaccine viruses in vivo. Although the ts-2 "revertants" were indistinguishable from wild type RS virus in high efficiency of plaque formation at 39°C and syncytium forming ability, none of the six "revertant" clones was virulent when evaluated in chimpanzees or owl monkeys. This suggests that true reversion by back mutation did not occur. The most likely explanation is that a modifying mutation occurred that restored the ts^+ phenotype and syncytium forming ability without restoring virulence.

Genetic stability of the ts-2 mutant in the chimpanzee is similar to that recorded for the attenuated poliovirus vaccine strains in man. "Revertant" virus, which has lost some or all of the temperature sensitivity of the vaccine virus (i.e., loss of the rct marker) or which has regained some neurovirulence for the monkey, is frequently recovered from the stools of vaccinees infected with attenuated poliovirus. However, such altered virus is rarely associated with paralytic disease in either vaccinees or their contacts; those rare individuals who develop paralytic disease are often immunodeficient. Indeed, it appears that vaccine virus or genetically altered vaccine virus which has lost some of its temperature sensitivity has replaced wild type poliovirus as the predominant virus circulating in the United Kingdom. Predominance of these viruses, however, is not associated with appreciable paralytic disease, suggesting that transmission of vaccine virus or partially altered vaccine virus may extend the benefits of immunization without significant risk to the community. ts-2 "revertants" may well behave in a similar manner. For this reason and because of its complete attenuation for both chimpanzees and owl monkeys, the ts-2 mutant should be evaluated in children for safety and for its usefulness in preventing RS virus disease of infancy and early childhood. (Richardson, Belshe, Chanock)

Pathogenesis - Experimental study of the basic mechanism of respiratory syncytial (RS) virus disease and the factors mediating recovery and immunity has been hampered by the lack of an appropriate small animal in which to investigate these questions. The recent description of RS virus infection in infant ferrets provided the first such model. However, the striking age dependence seen in pulmonary RS virus infection in ferrets, whereby infant animals rapidly become refractory to virus replication in the lung, disqualifies this model for extended studies of RS virus infection in the lower respiratory tract. In searching for an alternate experimental animal which would support RS virus replication in the lung at all ages, we found the cotton rat (Sigmodon hispidus) to be suitable. Subsequently the pathogenesis and immunology of RS virus infection were studied in these animals.

RS virus infection of cotton rats is an acute condition involving both upper and lower portions of the respiratory tract. Virus grows to high titer in nasal turbinates, infects a majority of epithelial cells and produces a moderately severe desquamative, exudative rhinitis. The trachea is relatively resistant to infection, virus grows to a low titer and does not induce any histologic changes. The lungs, like the nose, support viral replication to high titer. Infection, as monitored by immunofluorescence microscopy, is restricted to the bronchial and bronchiolar epithelium and is associated with mild bronchitis and bronchiolitis. The lungs of cotton rats are uniformly susceptible to infection throughout life. Continuous susceptibility of the cotton rats' lungs offers the possibility to explore certain aspects of RS virus pathogenesis previously not amenable to experimental study. First, the continuous susceptibility of the lungs to RS virus infection makes it possible to study the effect of vaccination upon resistance to infection. Use of this animal model may also permit elucidation of the mechanism by which formalin-inactivated, alum-precipitated RS virus vaccine potentiated, rather than protected against disease in young

children. Second, this model should permit an experimental study of the persistence of immunity following infection. In man it is clear that immunity to RS virus infection is transient and incomplete, but the reasons are not well defined. (Prince)

Immunology - The immunolgic response to respiratory syncytial (RS) virus infection has been measured in the past by the detection of a rise in complement-fixing (CF) or neutralizing antibodies in the convalescent serum of the infected individual. Failure to detect a rise in neutralizing or CF antibody in young infants during convalescence from severe respiratory disease caused by RS virus has been ascribed to immunologic immaturity of the infant or to the presence of preexisting maternal antibodies which mask an immune response or impair the ability of the infant to mount an immunologic response. A sensitive and rapid assay for serum antibodies to RS virus utilizing the enzyme-linked immunosorbent assay (ELISA) technique was developed with the expectation that it might be useful in studying the immunologic response of the young infant and that it would have wide applicability in diagnostic and seroepidemiologic investigations. Acute and convalescent phase sera from 91 infants and children hospitalized with RS virus lower respiratory tract disease were assayed by the CF, plaque reduction and ELISA techniques to define the relative efficiency of the three methods. Previously the diagnosis of RS virus disease in the study group had been established by virus isolation and/or a 4 fold or greater rise in serum CF antibody titer. In general there was concordance between the plaque reduction and ELISA techniques for detection of RS serum antibodies although the latter method usually yielded a higher titer. In the 1-3 month old age group RS virus was isolated from the oropharynx of each of the patients, yet only 1 of 49 developed a significant serum CF antibody rise to RS virus. Also only one of 47 young infants had a 4-fold or greater rise in plaque reduction neutralizing antibody. In contrast to the results obtained with these techniques, 25 of the 49 infants had a significant antibody rise when tested by ELISA, indicating the greater efficiency of the ELISA technique for detection of an RS virus serologic response during early infancy, the period when this virus is most important as a cause of serious lower respiratory tract disease. This greater efficiency of the ELISA technique was also evident to a lesser degree in the 4 to 6 month age group. In infants over 7 months old, the number of significant antibody rises detected by each of the 3 methods was similar. (Richardson Yolken)

INFLUENZA A VIRUS

New ts Mutants and Efforts to Genotype Older Mutants - Mutagenesis of A/Udorn/72 wild type virus yielded a series of new ts mutants some of which appear to belong to two new complementation groups. Mutants of the latter groups complement each other and the 6 standard Hong Kong complementation groups. This means that we now have mutants which represent each of the 8 viral genes. Availability of a complete set of ts mutants, representing each of the influenza viral genes, should facilitate genetic characterization of candidate ts mutant vaccine strains.

The influenza A virus genome is made up of 8 pieces of RNA that can be resolved by polyacrylamide gel electrophoresis (PAGE). Since the corresponding RNA segments from different strains may be induced to migrate at different rates by altering the conditions of electrophoresis, it is possible to determine the parental origin of genes present in recombinant viruses. Using this technique the genotype of a series of A/Vic/75 ts-1[E] recombinants was determined. It appears that genes from the wild type parent did not influence the level of attenuation of the A/Vic/75 ts-1[E] recombinants for adult volunteers. This interpretation is consistent with our previous observations indicating that the ts genes of ts recombinants are primarily responsible for the level of attenuation exhibited by such viruses. (Markoff, Shimizu, Thierry, Murphy)

Production and Characterization of ts-1A2 Recombinant Viruses -When immunity to both surface antigens is lacking, only the degree of defectiveness of the vaccine virus determines attenuation and in this situation defectiveness must be greater than that specified by the ts-1[E] ts mutations. For this reason we constructed a recombinant (1A2) with a set of ts mutations that specified a greater degree of defectiveness than that seen with the ts-1[E] recombinants. Initially ts mutants of wild type influenza A virus were produced by chemical mutagenesis. Next, viruses with a single ts lesion were identified among the ts progeny of mutagenized virus or were produced by segregating recombinants with single lesions from mutants bearing two or more ts lesions. The single ts mutants were characterized as to complementation-recombination group and were evaluated for degree of growth restriction and genetic stability in vivo (in the hamster). In this manner we identified the 2 most defective and stable ts lesions that were genetically distinct, i.e., located on different RNA segments of the genome. Finally, we combined the 2 lesions into a single virus by genetic recombination. The resulting recombinant virus (designated 1A2) that had the A/Udorn/72 hemagglutinin and the desired two ts lesions was more restricted in replication at 37°C than either ts parent or any of the ts-1[E] recombinants. Genetic evidence suggested that the 1A2 ts lesions were located on the genes coding for the P1 and P3 proteins; both of these large proteins are thought to be involved in the synthesis of complementary RNA. Thus, the 1A2 recombinant has defects in both proteins required for the initial step in viral replication.

The double ts-1A2 recombinant exhibited an impairment in complementation and recombination not found in two parallel passaged single ts viruses which possessed either the P1 or P3 ts gene present in ts-1A2 virus. Interestingly, the HK/68-ts-1[A] virus and its Udorn/72-ts-1[A] recombinants that possessed both P1 and P3 ts lesions did not manifest the impairment in complementation and recombinant seen with ts-1A2. This suggests that the P1 ts gene in the ts-1A2 virus differed from that in the HK/68 and Udorn/72 ts-1[A] viruses since the ts P3 gene in all of these viruses had a common origin. The reason for the impairment in complementation-recombination of the Udorn/72 ts-1A2 virus is not understood and can only be speculated upon. Possibly, many virions produced during a mixed infection involving the ts-1A2 virus are phenotypically mixed particles

possessing both the ts P1 and P3 polymerase proteins. Such viruses, irrespective of their genotype, would have greatly decreased infectivity at restrictive temperature because c-RNA synthesis from the virion RNA would be restricted by the ts P1 and P3 proteins. The presence of approximately 15 P1 and P3 proteins per virion suggests that each gene segment has one or more P1 and P3 proteins. Thus, such phenotypically mixed particles could be restricted at high temperature if any one of the 8 viral genes had both the ts P1 and P3 proteins attached to it. In this manner, the lack of recombination seen between the ts-1A2 virus and other ts viruses may reflect the presence of the ts-1A2 defective proteins in recombinant viruses whose genotype is $\frac{ts}{+}$.

Previously, it had been demonstrated that the extent to which ts recombinants were restricted in growth in the lower respiratory tract was a function of their temperature sensitivity as measured by shutoff temperature of plaque formation in tissue culture. The lower the shutoff temperature of a ts virus the lower the level of virus replication in the hamster's lungs. The A/Udorn/72 ts-1A2 virus, which had a 37°C shutoff temperature, failed to replicate in the lungs of hamsters and was significantly restricted in the nasal turbinates. Udorn/72-ts-1[E] recombinants, which had a 38°C shutoff temperature and ts lesions on genes coding for the P3 and NP proteins, replicated in the hamster's lungs to a level intermediate between that of the ts-1A2 recombinant and wild type virus and were not restricted in the nasal turbinates. This indicated that the Udorn/72-ts-1A2 virus (37°C shutoff) was more restricted in the upper and lower respiratory tract than its ts-1[E] counterparts (38°C shutoff). Although Udorn/72-ts-1A2 was restricted in replication in both the upper and lower portions of the respiratory tract, infection with this virus provided significant protection against wild type virus challenge.

Ideally a master strain which serves as the donor of attenuating genes should possess ts genes which are completely stable genetically in vivo. The P3 ts gene of the ts-1A2 recombinant appeared to meet this requirement, but the P1 ts gene exhibited some instability. However, together these two ts genes rendered the Udorn ts-1A2 recombinant genetically stable in vivo and thus the ts-1A2 genes may prove to be a satisfactory tandem set of ts lesions for use in a master strain.

Last year the A/Udorn/72-ts-1A2 double recombinant was mated with the A/Vic/3/75 wild type virus and the progeny were analyzed for shutoff temperature of plaque formation, ts genotype and level of replication in the respiratory tract of the hamster. In each instance transfer of the two ts-1A2 ts genes to an A/Vic/75 recombinant conferred upon that recombinant the properties of the A/Udorn/72-ts-1A2 parent. Thus, each A/Vic/75 ts-1A2 recombinant bearing both parental ts genes had a 37°C shutoff temperature for plaque formation, was completely suppressed in replication in the hamster's lungs, and was 100-fold restricted in growth in the hamster's nasal turbinates. Furthermore, each of over 100 isolates recovered from hamsters infected with one of the A/Vic/75 ts double lesion recombinants retained the ts phenotype.

The ability of the ts genes of the Udorn/72-ts-1A2 virus to reproducibly attenuate additional influenza A wild type viruses within the H3N2 subtype was evaluated this year by mating this donor with the A/Alaska/77 (H3N2) wild type virus. Each of the 4 Alaska/77-ts-1A2 recombinant viruses that possessed both ts-1A2 ts genes had a 37°C shutoff temperature. The 4 recombinants exhibited the same level of growth restriction in vivo as their Udorn/72-ts-1A2 parent; i.e., they were approximately 10,000-fold restricted in growth in the lungs and 100-fold in the nasal turbinates. Furthermore, each of the viruses recovered from hamsters infected with the A/Alaska/77 ts-1A2 recombinants retained the ts phenotype confirming the genetic stability of these lesions. Both ts P3 and ts P1 segregants were detected among the A/Alaska/77 recombinant viruses confirming that the Udorn/72-ts-1A2 parent indeed has two ts genes. Furthermore, our experience with the A/Alaska/77 recombinants provides additional evidence that ts-1A2 recombinant viruses that possess both ts-1A2 lesions exhibit a predictable set of genetic and biological properties.

The Udorn/72-ts-1A2 virus was also mated with the A/HK/123/77 (H1N1-Russian flu) virus and 3 recombinant ts-1A2 viruses that had both a 37°C shutoff temperature and the ts P1 and P3 genes were isolated. These recombinants resembled their A/Udorn/72 ts-1A2 parent with regard to restriction of replication in the nose and lungs of the hamster and genetic stability of the ts genes in vivo. These observations suggest that viruses within the H1N1 subtype can also be predictably attenuated by the acquisition of the two ts-1A2 lesions. (Murphy, Chanock)

Evaluation of A/Vic/75-ts-1A2 Recombinant Viruses In Adult Volunteers - An A/Victoria/75 ts-1A2 recombinant, that possessed ts mutations on the genes coding for the P1 and P3 proteins and that was markedly restricted in its replication at 37°C, was administered to 42 doubly seronegative volunteers at a dose of $10^{7.5}$ $TCID_{50}$. Prior to this trial we were concerned that the 1A2 recombinant might be overattenuated. Previously the A/Hong Kong/68 ts-1[A] recombinant, that had a 37°C shutoff temperature for plaque formation and ts mutations affecting the same proteins as the 1A2 recombinant, was found to lack infectivity for adults who had neuraminidase immunity but lacked hemagglutinin immunity. Nonetheless, the A/Victoria/75 ts-1A2 recombinant infected 90% of the volunteers who lacked serum antibodies for both the hemagglutinin and neuraminidase antigens. Only 5% of the volunteers developed mild coryza or rhinitis. Other signs or symptoms were not observed. Vaccinees shed virus for a shorter interval and at a lower titer than the Vic/75-ts-1[E] vaccinees. Each ts-1A2 isolate retained the ts phenotype indicating that the recombinant was stable genetically in doubly seronegative adults. Eighty six percent of the vaccinees developed a rise in serum HI antibody. These preliminary clinical data are extremely encouraging since they indicate that the A/Vic/75 ts-1A2 recombinant was more attenuated than the A/Vic/75 ts-1[E] recombinants. Further, the 1A2 recombinant was surprisingly antigenic considering its limited degree of replication. Finally, the ts-1A2 recombinant induced significant resistance to subsequent challenge with A/Vic/75 wild type virus. (Murphy, Markoff)

Evaluation of Cold-adapted Influenza A Recombinants in Adult Volunteers - Seronegative adult volunteers were administered A/Scotland/74 (CR18, clone 7) or A/Vic/75 (CR19) cold-adapted recombinants to assess their level of attenuation and antigenicity. In tissue culture the 2 viruses had a shutoff temperature for plaque formation of 39°C unlike their cold-adapted parent (PI-7) which had a shutoff temperature of 37°C. The A/Scotland/74 ca recombinant induced febrile or systemic reactions in some volunteers, whereas the A/Vic/75 ca recombinant appeared to be more suitably attenuated. These observations suggest that the ca phenotype of a recombinant influenza A virus may not always specify a satisfactory level of attenuation for man. (Murphy, Spring)

Role of Immunity to the Neuraminidase Antigen in Attenuation - As described last year the A/Vic/75-ts-1[E] recombinant clones 81 and 113 both bore the $H3_{75}N2_{75}$ surface antigens and retained some residual virulence for adults who lacked immunity to the $N2_{75}$ neuraminidase as well as the $H3_{75}$ hemagglutinin. Since lack of neuraminidase immunity was suggested as being a contributing factor to the reactogenicity of earlier ts-1[E] viruses in children, the importance of neuraminidase immunity in the reactogenicity of Vic/75 ts-1[E] recombinants was investigated. An A/Vic/75-ts-1[E] recombinant (clone 67) that bore the two ts-1[E] genes and the neuraminidase gene of HK/68-ts-1[E] ($N2_{65}$) was isolated. This virus was then evaluated in a group of young adults who lacked antibodies to $H3_{75}$ and $N2_{75}$ but who had moderate to high levels of $N2_{65}$ antibody. Clone 67 was considerably more attenuated than the Vic/75-ts-1[E] clone 81 and 113 recombinants that bore the $N2_{75}$ surface antigen. Vaccinees who received the clone 67 developed an anti-$H3_{75}$ response but not an anti-$N2_{75}$ response. By challenging such volunteers with A/Vic/75 ($H3_{75}$ $N2_{75}$) wild type virus it was possible to assess the contribution of hemagglutinin immunity to resistance to influenza A virus in the absence of neuraminidase immunity. Each of the vaccinees who developed a seroresponse to the $H3_{75}$ antigen following infection with clone 67 was protected against illness, but not infection when challenged with wild type $H3_{75}$ $N2_{75}$ virus. This indicates that hemagglutinin-specific immunity, like neuraminidase-specific immunity, can protect against illness without preventing infection. Formerly it had been thought that this was only a property of neuraminidase-specific immunity. (Markoff, Murphy)

GASTROENTERITIS

Important advances were made in elucidating the etiological agents of acute infectious nonbacterial gastroenteritis, a syndrome that affects a broad segment of the population.

Importance of Rotaviruses in the Etiology of Non-bacterial Gastroenteritis in Infants and Young Children - In studies carried out collaboratively with Children's Hospital, D.C., specimens from 593 inpatients with gastroenteritis were tested by EM and ELISA for viral agents and 236 (39.8%) were found to be positive for rotavirus. In contrast only 9

(1.8%) of 513 control inpatients studied were rotavirus positive by these techniques. Rotaviruses were detected in 43 (21%) of 201 outpatients with diarrheal disease and in 8 (2.7%) of 291 outpatient controls. Thus, rotaviruses cause not only severe diarrheal illnesses requiring hospitalization but also milder gastroenteritis illnesses which can be treated on an outpatient basis. The striking predelection of rotavirus infection during the cooler months of the year was a consistent finding. Adenoviruses (as detected by EM and/or tissue culture) were associated with only 4.9% of the diarrheal episodes; 1.9% of the controls were adenovirus positive. 27 nm particles were found only occasionally and as noted previously they do not appear to be important etiologic agents of infantile gastroenteritis.

A population of 24 infants and young children followed prospectively during the first three years of life in the Guatemalan highland village of Santa Maria Cauque was studied for the occurrence of rotavirus infection using enzyme-linked immunosorbent assay to detect virus in stools. Stool specimens were collected on a weekly basis from these individuals. Infection with rotavirus was associated with 26 (14.2%) of 183 selected diarrheal episodes during the 3 study years. Twenty of the 24 infants and young children had diarrhea associated with rotavirus on at least one occasion and 6 had 2 such episodes. The incidence of approximately 1.1 rotavirus diarrheal episodes per child during the first 3 years of life is an estimate based on selected diarrheal episodes excluding those associated with bacterial or parasitic pathogens (approximately 42%) and those from which stools were not available for study (approximately 5%). Although rotavirus was only associated with approximately 14% of selected diarrheal episodes in this survey, it accounted for a large proportion of severe diarrheal illness as defined by vomiting and dehydration. Rotavirus infection was associated with over 50% of the dehydrating diarrheal illnesses during the first 3 years of life. Also vomiting was 4 times more common among patients with rotavirus diarrhea compared to all other diarrheal episodes. (Kapikian, Wyatt, Yolken, Chanock)

Comparative Epidemiology of Rotavirus Serotypes - A major development in the epidemiologic study of rotavirus infections was the discovery of 2 rotavirus serotypes by workers in Belgium and England. Two-hundred thirty six rotavirus isolates were serotyped by a recently developed ELISA procedure. One-hundred seventy-five (74%) were type 2 and 61 (26%) type 1. Nontypable rotaviruses were not encountered. There was a marked fluctuation in the temporal distribution of rotaviral serotypes during the course of the study. Analysis of serotypes among both in and outpatients revealed that type 1 strains constituted 14% of the total from October, 1974 to September, 1976, 29% of the total in the October, 1976 to September, 1977 period, and 44% in the October, 1977 to May, 1978 period. In addition, an examination of shorter intervals within some of these periods revealed clustering of cases of one serotype or the other. For example, there were 20 illnesses associated with rotavirus infection during February, 1978. Ten of these infections occurred during the first half of the month, and all were identified as type 2 virus. On the other hand, 7 of the 10 rotavirus illnesses sampled during the second half of the month were associated with type 1 virus.

The proportion of type 1 and type 2 infections among outpatients and inpatients with gastroenteritis was similar. A difference in the mean age of patients infected with the 2 serotypes was not observed.

In the Children's Hospital studies, rotavirus isolates were distributed in an approximate 3:1 ratio of type 2 to type 1. Serotype identification was expanded to include study of rotavirus positive specimens obtained from other parts of the world--Guatemala, Africa, Costa Rica, Belgium, Asia, Australia and England. The overall frequency of type 1 and type 2 isolates among 136 rotavirus positive specimens was 18% and 82% respectively, a distribution not significantly different from that observed in the Washington, D.C. study.

The relative virulence of the 2 serotypes could not be determined from the Children's Hospital study because of its cross-sectional nature. However, in the Guatamalan cohort study, routine specimens were obtained on a bi-weekly basis as well as during episodes of gastroenteritis. An analysis of these samples revealed that each of the 16 type 2 strains were associated with diarrhea, whereas only 6 of the 11 type 1 strains were shed at the time of acute gastroenteritis. Five of the type 2 infections led to significant dehydration, while this condition was not observed during type 1 virus infection. Nine infants and children who had sequential rotavirus infection were studied, 7 in Guatemala and 2 in the U.S. Eight of these 9 individuals shed a different rotavirus serotype (type 1 or type 2) during the sequential episodes and type 1 caused the first infection in 8 of the 9 study patients. Significantly, type 1 virus infection did not protect against type 2 virus disease. One of the children had three rotavirus infections. All of the individuals who underwent type 2 infection were symptomatic, but only approximately one half of the type 1 infections caused disease.

Analysis of the prevalence of antibody to human rotavirus types 1 and 2 in 422 individuals indicated that infection with both serotypes occurred at an early age; by 18 months of age 85% of individuals had antibody to each serotype. A difference in prevalence of antibody to type 1 and type 2 was not statistically significant for any age group. This suggests that the clinical expression of type 2 virus infection is more pronounced than that of type 1 virus, an inference supported by observations made on individuals who underwent sequential rotavirus infections.

In other studies with paired sera from individuals shedding either type 1 or type 2 virus, it was observed that while there was some cross-reactivity, the immune response was largely directed against the rotaviral serotype responsible for infection.

These findings have implications for the development of methods for immunoprophylaxis of rotavirus associated gastroenteritis. One approach involves the use of a vaccine during infancy to induce active immunity. Vaccine could also be used during late pregnancy to boost colostral and milk antibodies in order to provide passive immunity to the nursing infant. The realization that there are 2 distinct human rotaviruses which do not appear to provide significant heterotypic immunity suggests

that to be completely effective a vaccine must induce resistance to both serotypes. (Yolken, Kapikian, Wyatt, Chanock)

Experimental Infection of Animals with Rotavirus - Various large animal species were infected experimentally in order to assess virulence of rotavirus strains derived from man and animals as well as to define relationships between selected rotavirus strains based on their ability to cross-protect. Studies were initiated with the plaque-purified "UK" strain of bovine rotavirus, which grows well in cell culture, to assess virulence of this strain in calves and piglets at Plum Island Animal Disease Center USDA, in collaboration with Dr. C.A. Mebus. This information is needed in order to obtain clearance from the USDA for further study of this virus in the U.S. The "UK" strain has been propagated under conditions which make it acceptable for evaluation in man as a candidate vaccine strain. The concept of cross-protection between heterologous strains of rotavirus from different species was evaluated in the newborn gnotobiotic calf. Calves rapidly lose their susceptibility to the human rotavirus so that challenge must be performed shortly after birth. Thus, in order to evaluate the protective effect of calf rotavirus against the human virus it was necessary to infect calves in utero with the former virus and perform challenge with the human virus shortly after birth. In sequential challenge studies (performed in collaboration with Dr. Mebus) initial infection in utero with bovine rotavirus induced complete resistance to disease when calves were challenged at birth with the human rotavirus. Thus these viruses appear to be sufficiently related for the bovine virus to induce immunity to the human rotavirus. (Wyatt)

In Vitro Cultivation of Rotaviruses - While the human rotavirus has not been grown to a high titer in tissue culture, several animal rotaviruses have been cultivated successfully. Recently, it was reported that cultivation of a Canadian calf rotavirus and a U.S. pig rotavirus was significantly enhanced by incubating trypsin, chymotrypsin, or pancreatin with virus prior to inoculation of tissue culture. We have confirmed this effect and have seen a 10-32 fold increase in infectivity, as measured by plaque assay, following exposure of bovine rotavirus to trypsin. Unfortunately, the optimal conditions of trypsin enhancement that increased infectivity of the bovine rotavirus did not have a similar effect upon the human rotavirus. (Wyatt)

Enhancement of Antigen Incorporation and Infection of Cell Culture by Human Rotavirus - Three factors were found to be of importance in infectivity of human rotavirus for tissue culture. First, aggregation of virus particles appeared to be a pre-requisite for infection. Mild sonication (insufficient in itself to reduce the titer of infectious virus) followed by filtration through a 450 nm, filter (which did not result in any loss of total antigenicity as determined by radioimmunoassay) caused a precipitous drop in infectivity as compared to untreated specimens. In contrast, a decrease in infectivity with the tissue culture-adapted simian rotavirus (SA-11) was not seen and only a mild drop of infectivity with an in-vitro cultivable calf rotavirus was noted when these viruses were treated in a similar way. Second, low-speed centrifugation was

found to be an important factor in infectivity. Without centrifugation, human rotavirus failed to infect monolayers. Third, incorporation of trypsin in the inoculum to a final concentration of 10 ug/ml enhanced infectivity of the human strains, but not the calf or simian strains tested. This enhancement of infectivity appeared to be an effect on the virus rather than on the tissue culture cells, as pre-treatment of cells did not increase infection, whereas pre-treatment of virus enhanced infectivity. Serial passage of monolayers infected by human virus under conditions described above revealed that viral antigen was detectable by immunofluorescence and ELISA up to the third passage, although the percentage of infected cells decreased considerably with each passage. Infectivity of cell cultures and in vitro human rotavirus protein formation was demonstrated by ^{35}S methionine incorporation, and the specificity of this de-novo synthesized viral material was established by radio-immunoprecipitation. (Schoub)

Characterization of Rotavirus RNA and Comparison of Rotavirus Strains by Gel Electrophoresis of Viral RNA - Coelectrophoresis comparisons of human strains revealed 3 different patterns which formed the basis for grouping them. Group I and Group II differed in the migration of segment no. 5. Groups I and II differed from Group III in the migration of segments no. 6, 9, and 10. Comparisons among the animal strains resulted in a pattern with differences in the migration of 3 or 4 segments, usually in the largest sized RNA class. When the RNA's from the human and animal strains were coelectrophoresed differences in the migration of 5 or 6 segments resulted and these differences were in all 4 size classes of RNA. In comparisons of human viruses with animal strains, frequent differences were observed in the migration of the 3rd, 4th, 5th, 9th and 10th segments. For these reasons gel electrophoresis of RNA has proved to be a sensitive technique for identifying rotaviruses following in vivo or in vitro propagation and may prove useful for studying natural transmission of rotaviruses between species.

Gel electrophoresis of viral RNA should prove valuable in genetic studies with rotaviruses, particularly in establishing the genotype of reassortants ("recombinants"). (Kalica)

Role of Norwalk Virus in Outbreaks of Non-Bacterial Gastroenteritis -The development of a radioimmunoassay (RIA) for detection of Norwalk viral antigen and a radioimmunoassay blocking (RIABL) test for detection of antibody to the Norwalk agent as well as the development of an immune adherence hemagglutination assay (IAHA) for detection of purified high titered Norwalk antigen and of Norwalk antibody were developed in order to facilitate epidemiologic studies of this virus and to define its role in acute gastroenteritis. Progress was made in studying the natural history of Norwalk virus infection by examining paired sera from a series of 25 outbreaks of gastroenteritis. In most outbreaks the well established causes for epidemic diarrhea such as Shigella and Salmonella had been excluded. In two outbreaks acute phase stool specimens were available and examined by RIA for the presence of Norwalk antigen.

Six (26%) of the 23 newly studied outbreaks appeared to be associated with the 27 nm Norwalk virus. Two of the outbreaks occurred on a college campus, 2 involved cruise ships, one involved a school in the Washington, D.C. area and one occurred in Japan. In two previously described outbreaks, the original Norwalk, Ohio epidemic and the Montgomery County outbreak, an association with the Norwalk virus had been demonstrated by studies in volunteers as well as by IEM study of paired sera derived from each outbreak. In an additional three outbreaks a small proportion of individuals had a seroresponse to the Norwalk agent. These rises, while significant, tended to be of lesser magnitude and occurred less frequently than in the outbreaks described above.

Thus, in this preliminary survey Norwalk virus or a Norwalk related virus appeared to account for a substantial percentage (26%) of epidemic acute infectious non-bacterial gastroenteritis. Also, the serologic evidence of infection on two cruise ships and in Japan confirms our previous finding that Norwalk virus infection is not limited to the United States. If one includes the original Norwalk outbreak as well as the serologically related Montgomery County outbreak, 8 separate outbreaks of Norwalk gastroenteritis were identified among 25 outbreaks studied (32%). It is possible that a majority of undiagnosed non-bacterial gastroenteritis might have an assignable etiology if satisfactory serologic assays existed for the antigenically distinct 27 nm Hawaii and "W" viruses which share biophysical and epidemiologic properties with the Norwalk virus. (Greenberg, Kapikian)

MYCOPLASMA

Efforts to assess the significance of pathogenic spiroplasmas are centered around the biological and serological characterization of organisms within this group, on development of appropriate models to explore pathogenicity and host response, and on evaluation of various procedures to determine the possible role of these agents in human disease. A number of new tick-derived pathogenic spiroplasmas have now been isolated and examined in the laboratory, offering confirmation of the value of the culture medium developed here for the primary recovery of these agents. Biophysical and serological characterization of the spiroplasmas pathogenic for vertebrates now in progress would suggest that these organisms are completely distinct from previously described plant and insect spiroplasmas. Pathogenicity tests in primates gave variable results, possibly related to pre-existing antibody to the challenge organism in some of the test animals. Further tests are in progress involving challenge of other primates, including challenge of younger animals and more lengthy holding of infected animals to assess long-term consequences.

Sero-epidemiologic studies using complement-fixation have focused on paired sera from children with unclassified central nervous system disease. Four-fold or greater antibody responses to the suckling mouse cataract agent (SMCA) antigen were observed in some of these children, although further testing of additional individuals is required to establish statistical significance. There was no evidence that the SMCA spiroplasma was involved in Reye's syndrome or in Lyme arthritis of children, although

further efforts at primary isolation of spiroplasmas from children with these diseases are in progress. A new serological test, defined as the "Deformation/Metabolic Inhibition" procedure has been developed in this laboratory. This procedure provides excellent separation of individual spiroplasmas and the test is now being applied to sero-epidemiologic studies of a number of animal hosts. (Tully)

Administrative or Organizational Changes

There have been no administrative or organizational changes made during the past year within the Laboratory of Infectious Diseases

Honors and Awards

Robert M. Chanock

Vice Chairman, Panel I, NIAID Virology Task Force
Director, International Reference Laboratory for Respiratory Virueses other than Influenza, World Health Organization, 1973-
Director, International Reference Laboratory for Mycoplasmas, World Health Organization, 1973-
Editorial Board, Journal of Infectious Diseases
Associate Editor, American Journal of Epidemiology
Member, Advisory Board Archives of Virology
Chairman, Section on Medical Microbiology and Immunology, National Academy of Sciences
Member, Bilaws Committee, National Academy of Sciences, 1978
Elected Member, Class Membership Committee, National Academy of Sciences, 1978
Recipient, Honorary degree of Doctor of Science, University of Chicago, at 50th Anniversary Convocation of the University of Chicago Medical School, November, 1977
Dyer Lecturer, National Institutes of Health, May 31, 1978
Chairman, Coronavirus Workshop, Fourth International Congress for Virology, The Netherlands, August, 1978
Invited speaker, Colloquium on Selected Diarrheal Diseases of the Young, NIH, March, 1978

Albert Z. Kapikian

Invited member, International Coronavirus Study Group, 1972--
Invited member, NIAID task Force in Virology, 1976--
Invited member, Study Group on Reoviridae of International Committee for the Toxonomy of Viruses, 1976--
Member, NIAID, Ad Hoc Panel for promotions from GS-9 to GS-11 level, 1975-1978
Invited member, Rotavirus subgroup of the WHO/FAO Comparative Virology Reoviridae Group, 1977--
Invited participant, Subcommittee on Interactions of Infection and Nutrition Board, National Academy of Sciences Workshop on Effective Interventions to Reduce Infections in Malnourished Populations", Port-au-Prince, Haite, June, 1977
One of two Vice Chairmen, Workshop "Aspects of Gastroenteritis Virus" at the Fourth International Congress for Virology, The Hague, Netherlands, August, 1978
Invited lecturer, Tropical Medicine Course, Walter Reed Army Institute of Research, July, 1978
Invited speaker, children's Hospital National Medical Center, Infectious Disease Conference, December, 1977
Invited to serve as moderator of a session at the Colloquium on Selected Diarrheal Diseases of Young Animals and Humans, Bethesda, Md., March, 1978
Invited participate in Workshop on New and Useful Methods in Viral Diagnosis at NIH, Bethesda, January, 1978

Albert Z. Kapikian - continued

Invited participant to Workshop on Research in Digestive Diseases, NIH January, 1978
Invited lecturer at FAES Class on Introductory Virology, NIH, May, 1978
Invited participant, Consultation on Immunity and Vaccine Development Against Enteric Infections, WHO, Geneva, August, 1978

Robert H. Purcell

Recipient of Gorgas Medal, Nov., 1977
Recipient, Distinguished Service Medal, April, 1978
Invited Participant, "Clinical Day": New Frontiers in Hepatitis, Montreal, Oct. 19, 1977.
Invited Speaker, Amer. Soc. Clin. Path., Las Vegas, Oct. 26, 1977.
Invited Speaker, American Association for the Study of Liver Diseases, Chicago, Nov. 1-2, 1977.
Participant, International Workshop on Hepatitis B and Liver Cancer, NCI, Bethesda, Dec., 1977.
Invited Speaker, American College of Physician's Course, March 29-31, 1978, Rochester, Minnesota, Mayo Clinic.
Invited Speaker, ICN-UCLA Symposium on Persistent Viruses, Keystone, Colo., March 13-17, 1978.
Invited Speaker, Second U.C.S.F. Symposium on Viral Hepatitis, San Francisoco, California, March 16-19, 1978.
Invited Participant, "Task Force on Use and Needs for Chimpanzees"., NIH, May 22, 1978.
Invited Participant, Workshop on Research in Digestive Diseases. National Commission on Digestive Diseases. January 16-17, 1978.

Joseph G. Tully

Invited speaker Tissue Culture Association, Denver, Colorado, June, 1978
Invited speaker, International Organization for Mycoplasmology, 2nd Conference, Freiburg, Germany, August, 1978
Chairman, Symposium on pathogenicity of mycoplasmas for man, animals, plans and insects. XII International Congress of Bacteriology, Munich, Germany, September, 1978
Participant and Chairman, WHO/FAO Board on Comparative Mycoplasmology
Chairman, International Organization for Mycoplasmology
Chairman, Subcommittee on Mycoplasma Taxonomy, American Society for Microbiology
Member, International Subcommittee on the Taxonomy of Mycoplasmatales
Co-director, Workshop on detection of mycoplasmas, Cell Culture Center, Lake Placid, New York, November, 1977

Brian R. Murphy

Invited speaker, the Symposium on the Advances in Vaccination Against Virus Diseases, Berne, Switzerland, June, 1978
Invited participant, Fourth International Congress for Virology, The Netherlands, August, 1978

Richard G. Wyatt

Invited speaker, Workshop on New and Useful Methods in Viral Diagnosis, NIH, January 12-13, 1978
Member, U.S. Biomedical Research Team to Egypt, January 15-29, 1978 to explore establishment of research projects in priority areas.
Member of Joint Working Group on Enteric and Diarrheal Diseases
Member Program Committee for American Veterinary Medical Association Colloquium on "Selected Diarrheal Diseases of the Young", NIH, March 7-9, 1978
Invited to give presentation on "Viral Diarrheas" for Special Studies: Medical Virology in the Tropics, April 26, 1978, Tulane University, School of Public Health and Tropical Medicine, New Orleans, LA.
Invited speaker, Workshop on Aspects of Gastroenteritis Viruses, Fourth International Congress for Virology, The Hague, Netherlands, August, 1978

Stephen M. Feinstone

Invited speaker, Hepatitis Viruses Symposium, University of California School of Medicine, San Francisco, March, 1978
Invited speaker, Tenth International Course on Transplantation and Clinical Immunology, Lyon, France, May, 1978

Harry B. Greenberg

Invited speaker, Colloquium on Selected Diarrheal Diseases of the Young, NIH, March, 1978
Invited speaker, Aspects of Gastroenteritis Viruses Workshop, Fourth International Congress for Virology, The Hague, Netherlands, August, 1978
Recipient, Public Health Service Commendation Medal, June, 1978

Susan B. Spring

Invited to present a seminar on Influenza Vaccines at the University of Chicago, Department of Microbiology, August, 1978

Robert H. Yolken

Invited Scientist, Workshop on Diarrheal Diseases, Cholera Research Center, Calcutta, India, February, 1978
Invited participant, American Society of Microbiology Conference on Enzyme Immunology, Atlanta, May, 1978

Anthony R. Kalica

Invited speaker, Colloquium on Selected Diarrheal Diseases of the Young, NIH, March, 1978
Invited speaker, Aspects of Gastroenteritis Viruses Workshop, Fourth International Congress for Virology, The Hague, The Netherlands, August, 1978

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00021-09 LID

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
THE ETIOLOGY OF ACUTE INFECTIOUS NONBACTERIAL GASTROENTERITIS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Albert Z. Kapikian	LID, NIAID	Head, Epidemiology Section
	Dr. Harry B. Greenberg	LID, NIAID	Medical Officer
	Dr. Anthony R. Kalica	LID, NIAID	Research Microbiologist
	Dr. Barry S. Schoub	LID, NIAID	Guest Worker
	Dr. Richard G. Wyatt	LID, NIAID	Medical Officer
	Dr. Robert H. Yolken	LID, NIAID	Research Associate
Other:	Dr. Robert M. Chanock	LID, NIAID	Chief
	Mr. W. Lee Cline	LID, NIAID	Bio. Lab. Tech. (Micro)

COOPERATING UNITS (if any)
See next page

LAB/BRANCH
Laboratory of Infectious Diseases

SECTION
Epidemiology Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
164.5/12	60/12	104.5/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: (1) To search for viruses which play an etiological role in the syndrome of acute infectious non-bacterial gastroenteritis of infants, children, and adults; (2) to cultivate (in vitro) the viral agents of acute infectious nonbacterial gastroenteritis; (3) to study the biophysical, immunological and epidemiological characteristics of such agents; (4) to reproduce the syndrome experimentally for the purpose of a) studying the pathophysiological and immunological responses of the host, and b) assaying the infectivity of viruses such as human rotaviruses and the Norwalk and Norwalk-like agents; (5) to develop effective immunoprophylaxis for the viruses of acute, infectious, nonbacterial gastroenteritis; (6) to develop efficient and sensitive assays for a) detection of the viral and bacterial agents associated with gastroenteritis and b) antibodies to these pathogens.

PHS-6040

Cooperating Units

Johns Hopkins University, Baltimore; Center for Disease Control, Atlanta; Meloy Laboratories, Rockville; Plum Island Animal Disease Center, USDA, New York; New York Blook Center; University of Minnesota School of Medicine; University of Massachusetts; Unversity of Maryland; University of Virginia, Charlottesville; Bureau of Biologics, FDA; NINCDS; Veterinary Resources Branch, DRS; Cholera Research Laboratory, Dacca, Bangladesh; Royal Children's Hospital, Melbourne, Australia; Fairfield Hospital; Melbourne, Australia; INISA, San Jose Costa Rica; INCAP, Guatemala; University of Nairobi, Kenya; Tokyo University, Sendai, Japan; Dept. Veterinary Science, Ohio Agricultural Research and Development Center, Wooster, Ohio; LBV, NIAID; LSD, NIAID.

Major Findings

Background: Important advances have been made in elucidating the etiological agents of acute infectious nonbacterial gastroenteritis, a syndrome that affects a broad segment of the population. It appears that viral gastroenteritis consists of at least two entities with distinct epidemiological characteristics. One, designated epidemic viral gastroenteritis, tends to occur in family or community-wide outbreaks affecting adults, school-aged children, family contacts and probably young children as well. The illness is usually self-limited and characteristically lasts 24-48 hours. In 1972, we demonstrated by immune electron microscopy (IEM) that a 27 nm particle--the Norwalk agent--was associated with an outbreak of this form of gastroenteritis that occurred in Norwalk, Ohio. In addition, particles that resemble the Norwalk agent morphologically have also been associated by IEM with 2 family outbreaks of gastroenteritis, one in Hawaii and the other in Montgomery County (MC), Md. The Norwalk and Hawaii agents appear to be distinct, whereas the Norwalk and MC agents appear to be related.

The other form of gastroenteritis designated sporadic infantile gastroenteritis has been associated predominantly with a severe form of diarrhea affecting infants and young children, that often necessitates hospitalization and parental fluid therapy. The illness rate among family contacts of infants with this form of diarrhea is low. It is evident now from studies in many parts of the world that the 70 nm, human rotavirus is a major etiological agent of sporadic infantile gastroenteritis. Before the discovery of this agent by Australian investigators in 1973, the etiology of the majority of cases of infantile gastroenteritis was unknown.

Our gastroenteritis studies are focused on the rotaviruses as well as on the Norwalk group of viruses. Significant progress has been made in developing rapid, efficient and sensitive methods for the detection of infections caused by these viruses.

Studies on the Etiology of Non-Bacterial Gastroenteritis Among Infants and Young Children Admitted to Children's Hospital, Washington, D.C. - These studies are carried out collaboratively with Children's Hospital. Currently the stool or rectal swab specimens are examined by EM and ELISA at Children's Hospital for gastroenteric agents (Dr. Brandt) whereas the routine rotavirus serologic studies (CF) are carried out at the LID (Mr. Cline). The clinical data and specimens are furnished by Dr. Kim and her staff at Children's Hospital. In addition, the serotyping of rotaviruses--a new development since last year's annual report is performed at LID (Dr. Yolken). The scope of the study has been outlined previously and essentially involves study of pediatric patients admitted to Children's Hospital with a diarrheal illness. Limited outpatient studies are also performed.

In data analyzed by Dr. Brandt the importance of rotavirus as the major etiologic agent of diarrheal illness is highlighted. From January, 1974 to May, 1978, specimens from 593 inpatients with gastroenteritis were

tested by EM and ELISA for rotavirus viral agents and 236 (39.8%) were found to be positive. In contrast only 9 (1.8%) of 513 control inpatients studied March, 1974 to May, 1978 were rotavirus positive by identical techniques. The striking predelection of rotavirus infection during the cooler months of the year was a consistent finding. Adenoviruses (as detected by EM and/or tissue culture) were associated with only 4.9% of the diarrheal episodes; 1.9% of the controls were adenovirus positive. 27 nm particles were found only occasionally and as noted previously they do not appear to be important etiologic agents of infantile gastroenteritis.

From November, 1975-May, 1978, rotaviruses were detected in 43 (21%) of 201 outpatients with diarrheal disease and in 8 (2.7%) of 291 outpatient controls. Thus, it appeared that rotaviruses caused not only severe diarrheal illnesses requiring hospitalization but also milder gastroenteritis illnesses which could be treated on an outpatient basis.

A major development in the epidemiologic studies of rotavirus infections involves the discovery of at least 2 serotypes of rotaviruses by workers in Belgium and England. All 236 rotavirus isolates have been serotyped by ELISA; 175 (74%) were type 2 and 61 (26%) type 1. Nontypable rotaviruses were not encountered. There was a marked fluctuation in the temporal distribution of rotaviral serotypes during the course of the study. Analysis of serotypes among both in and outpatients revealed that type 1 strains constituted 14% of the total from October, 1974 to September, 1976, 29% of the total in the October, 1976 to September, 1977 period, and 44% in the October, 1977 to May, 1978 period. In addition, an examination of shorter intervals within some of these periods revealed clustering of cases of one serotype or the other. For example, there were 20 illnesses associated with rotavirus infection during February, 1978. Ten of these infections occurred during the first half of the month, and all were identified as type 2 virus. On the other hand, 7 of the 10 rotavirus illnesses sampled during the second half of the month were associated with type 1 virus. The proportion of type 1 and type 2 infections among outpatients and inpatients with gastroenteritis was similar. A difference in the mean age of patients infected with the 2 serotypes was not observed.

Sequential Enteric Illnesses Associated with Different Rotavirus Serotypes In a Single Patient - We collaborated with Children's Hospital investigators to determine the rotavirus serotypes responsible for sequential enteric illnesses in a rotavirus-infected child who had 2 attacks of gastroenteritis 11 months apart, both of which were associated with rotavirus infection. On initial admission at 12 weeks of age, the child was acutely ill with evidence of dehydration and acidosis. During the first illness type 1 rotavirus was detected in the stool and a serologic response to this serotype was demonstrated by ELISA. Eleven months later, the patient developed another, but milder episode of enteritis and was admitted in part for social reasons. This time, however, rotavirus type 2 was detected in the stool. Paired sera were not available from the second hospitalization; however, antibody to type 1 persisted at a high level for 11 months after

the first illness, whereas antibody to type 2 was not detectable during the acute phase of the second illness. Whether concomitant infection with an E. coli which produced a heat labile enterotoxin contributed to the second illness is not known. The occurrence of sequential infection by 2 distinct rotavirus serotypes within 11 months suggests that infants and children may be at greater risk of rotavirus disease than had been appreciated previously. A definition of the epidemiology and clinical importance of these (and possibly additional) serotypes is of the highest priority, especially for the development of an effective strategy for immunoprophylaxis.

Comparative Epidemiology of Human Rotavirus Types 1 and 2 As Studied by ELISA - As noted above, in the Children's Hospital studies, rotavirus isolates were distributed in an approximate 3:1 ratio of type 2 to type 1. Serotype identification was expanded to include study of rotavirus positive specimens obtained from other parts of the world--Guatemala, Africa, Costa Rica, Belgium, Asia, Australia and England. The overall frequency of type 1 and type 2 isolates among 136 rotavirus positive specimens was 18% and 82% respectively, a distribution not significantly different from that observed in the Washington, D.C. study.

The relative virulence of the 2 serotypes could not be determined from the Children's Hospital study because of its cross-sectional nature. However, in the Guatamalan cohort study, routine specimens were obtained on a bi-weekly basis as well as during episodes of gastroenteritis. An analysis of these samples revealed that all 16 type 2 strains were associated with diarrhea, whereas only 6 of the 11 type 1 strains were shed at the time of acute gastroenteritis (p .05). Five of the type 2 infections led to significant dehydration, while this condition was not observed during type 1 virus infection (p 0.05). We also had the opportunity to determine the serotype of rotaviruses detected in stools from 8 asymptomatically infected newborns in a nursery in England. These newborns were in the nursery at a time when pediatric patients were hospitalized with rotaviral gastroenteritis in a nearby ward. The 8 rotaviruses from the nursery group as well the rotaviruses recovered from the hospitalized gastroenteritis patients were each type 2. Thus, type 2 virus is capable of causing asymptomatic infection in the normal newborn. The frequent occurrence of asymptomatic infection during the newborn period is one of the currently unexplained aspects of rotavirus epidemiology.

In addition to the single sequential rotavirus infection described above, 8 additional sequential infections were observed, 7 in Guatemala and one in the U.S. It was noteworthy that 7 of these 8 children shed a different rotavirus serotype (type 1 or type 2) during the sequential episodes and that type 1 caused the first infection in 8 of the 9 sequential episodes. Type 1 virus illness did not protect against type 2 virus disease. One of the children had three rotavirus infections. Most but not all of the infections observed in these children were symptomatic.

Analysis of the prevalence of antibody to human rotavirus types 1 and 2 in 422 individuals 6 months to approximately 65 years of age indicated that

infection with both serotypes occurred at an early age; by 18 months of age 85% of individuals had antibody to each serotype. A difference in prevalence of antibody to type 1 and type 2 was not statistically significant for any age group. In addition, there was no year to year variation in prevalence of antibody to either serotype among individuals 6 months to 3 years of age tested over the period 1974 through 1977. While only 24% of the isolates from symptomatic children from the Washington, D.C. area over a 5 year period were type 1, seroepidemiologic studies revealed that 85% of individuals had detectable antibody to both serotypes early in life suggesting that the clinical expression of type 2 virus infection is more pronounced than that of type 1 virus. Observations made during the cohort study of Guatamalan children supports this suggestion as infection with type 1 was asymptomatic in 45% of instances, while every type 2 infection was associated with gastroenteritis. This postulated greater virulence of type 2 human rotavirus is similar to that observed previously for type 1 poliovirus which exceeds the type 2 and type 3 polioviruses in neuropathogenicity.

In additional studies with paired sera from individuals shedding either type 1 or type 2 virus, it was observed that while there was some cross-reactivity, the immune response was largely directed against the rotaviral serotype responsible for infection.

IgA antibody to the type 1 and type 2 rotaviruses was examined in 43 colostral and 64 milk specimens from women living in Bangladesh, Guatemala, Costa Rica and Washington, D.C.; 88% of the colostral specimens had IgA anibody to type 1 rotavirus and 91% to type 2. Although the geometric mean titer to each serotype was significantly lower in milk than in colostrum, 88% of the milk specimens had antibody to type 1 and 88% to type 2. Only 2 specimens (both milk) lacked detectable antibody to both serotypes.

The ability of a nursing mother to respond to rotaviral infection with an increase in antibody in breast milk was documented in a woman who was infected with type 1 rotavirus at the time her infant underwent infection with this virus. In this instance a 64-fold increase in milk antibody titer to type 1, without an accompanying type 2 response, was observed.

These studies have important implications for the development of methods for immunoprophylaxis of rotavirus associated gastroenteritis. One approach involves the use of a vaccine during infancy to induce active immunity. Vaccine could also be used during late pregnancy to boost colostral and milk antibodies in order to provide passive immunity to the nursing infant. The realization that there are 2 distinct human rotaviruses which do not appear to provide significant heterotypic immunity suggests that to be completely effective a vaccine must induce resistance to both serotypes.

Common Exposure Outbreak of Type 2 Rotavirus Gastroenteritis With High Secondary Attack Rate Within Families - During February, 1978, an outbreak of acute gastroenteritis occurred in a playgroup which met once a week at the home of one of the participants. The group consisted

of 9 children, 15 months through 5 years of age, belonging to five families in suburban Virginia. Each of the 9 developed gastroenteritis which was characterized by sudden onset of vomiting and diarrhea. Three of the 5 mothers who shared in the playgroup activities also developed gastroenteritis. Four of the 5 fathers and both grandmothers in these families also developed diarrhea although they did not participate in the playgroup's activities. Thus, all 9 playgroup members, 7 of 10 parents and both grandparents, or a total of 18 of 21 exposed individuals, developed gastroenteritis with diarrhea, vomiting and fever or a combination of these within a period of about 1 week. The probable index cases were 2 non-playgroup siblings aged 15 months and 4 years. A playgroup mother had taken care of these children along with her own 2 playgroup children about 2 days before the weekly playgroup session. The 2 suspected index cases had experienced gastroenteritis during the previous week and were still symptomatic. The playgroup mother who cared for the index cases experienced diarrhea 24 hours before the playgroup meeting; in addition, her 3 yr. old daughter had onset of diarrhea just before the playgroup met and vomited while participating in the activities of the playgroup. The illness spread rapidly among playgroup members and their contacts and appeared to have an incubation period of 24-48 hours.

Stools were available from 11 of 21 study individuals. Rotavirus was detected in at least one stool specimen from 5 of the children and one of the symptomatic adults; thus 6 (55%) of the 11 shed rotavirus. Each of the 6 rotavirus strains was found to be type 2 by ELISA. Each of the 4 children and 3 adults from whom paired sera were obtained developed a 4-fold or greater antibody rise to type 2 by ELISA. One adult who developed a seroresponse but failed to shed detectable rotavirus did not report symptoms of gastroenteritis. Overall, it was possible to demonstrate evidence of human rotavirus infection by detection of virus and/or demonstration of a seroresponse in 10 of 11 individuals whose samples were available for testing.

Thus, illness within the playgroup progressed mainly from the highly susceptible young child to presumably partially immune parents and grandparents, indicating that rotavirus infection and illness may occur at all ages. The situation observed within the playgroup may well represent one model for spread of human rotavirus within the community. The high frequency of symptoms among adults was unexpected in view of our previous experience with adult contacts of infected pediatric patients as described in last year's annual report.

Enzyme Linked Immunosorbent Assay for Identification of Rotaviruses from Different Animal Species and From Man - Rotaviruses infect a wide variety of animals having been documented in calves, piglets, foals, rabbits, lambs, deer, antelope and the monkey. Different members of the rotavirus group are similar in appearance morphologically and they cross-react in a variety of immunological tests. Several species of newborn animals are susceptible to infection with human rotavirus; however, the role of animal rotaviruses in human disease and the exchange of rotaviruses among animals and man is not known. Studies in this area have been hampered by the lack

of a single method for distinguishing the rotaviruses derived from different species. A blocking ELISA was developed for this purpose to distinguish members of the rotavirus group. This assay is analogous to the classical tissue culture neutralization assay in that antigen and antibody are incubated together and unbound antigen is assayed. However, in this assay system, ELISA is used to measure the amount of unneutralized antigen. In this manner, rotavirus obtained from one animal species was found to be neutralized to a greater extent by homologous antibody than by antibody against rotavirus derived from other host species.

Thirty-three rotavirus isolates from children living in three different parts of the world could not be differentiated by this technique, but they were distinct from 4 strains recovered from calves, and a series of strains isolated from piglets, foals, monkeys and infant mice. The four bovine strains were similar, but they could be differentiated from the other animal strains, each of which exhibited a distinct pattern when tested by the ELISA blocking technique. In addition, a serum with antibody to an unknown rotavirus can be tested against a panel of agents with known specificity to determine which virus gave rise to the antibody. The assay is applicable regardless of the animal from which a particular virus strain or antiserum is derived. Specificity was noted only with serum containing antibody induced initially or solely by infection. Serums obtained from animals only immunized parenterally with antigen prepared by conventional techniques reacted equally well with all of the rotaviruses. This suggests that such serums contain large amounts of antibody directed against common viral determinants, whereas serums collected after gastrointestinal infection, whether boosted by parenteral inoculation of antigen or not, contain antibody directed primarily against specific determinants.

The observation that rotavirus infection occurs in a variety of animal species as well as man has raised the possibility of human to animal or animal to human transmission. In the past, technical difficulties have prevented large scale epidemiological studies to answer this question. This question can now be approached in a systematic manner using the ELISA blocking technique.

Modification of ELISA for Rotavirus Antigen Detection - In last year's annual report we described the development of a solid phase direct ELISA which was sensitive and efficient for detection of human rotavirus. In this test guinea pig anti-human rotavirus globulin was employed both as the precoat and the second antibody labelled with enzyme, in other words a direct sandwich ELISA.

After publication of our article describing this ELISA method we received many requests for rotavirus antiserum. To generate a large volume of this reagent, we immunized a goat. While this serum can be conjugated and used as in our original technique, a more sensitive test can be performed using a three-antibody ELISA similar to that employed in our detection system for heat-labile E. coli toxin. After pretreatment of wells with goat anti-rotavirus antibody and addition

of the stool specimens to be tested, an unlabelled guinea pig anti-rotavirus antibody is added, followed by an alkaline-phosphatase-labelled goat anti-guinea pig globulin. Since the anti- rotavirus reagents do not have to be conjugated they can be used highly diluted (1/3000 to 1/100,000) thus extending these valuable sera. In addition, because of the amplification effect of using an additional antibody, the color reaction is stronger, allowing for easier visual identification of positive specimens.

Using this method we have examined a large number of stools from the United States and Central America for rotavirus. However, when we examined stools from Bangladesh we noted that many gave a positive ELISA reaction although they did not contain rotavirus. Further investigation revealed that these false-positive reactions were caused by anti-goat antibodies in the stools, presumably due to the widespread consumption of goat milk. Such anti-goat antibodies react with the goat serum pre-coat and the goat anti-guinea pig globulin producing a false-positive reaction. This activity can be blocked totally by diluting stool in 1% goat serum which is free of rotavirus antibody prior to performing the ELISA. After dilution in goat serum the false-positive reaction does not occur. Using this modified technique, 183 stools have been studied successfully for the presence of rotavirus.

Immunological Response to Infection with Human Rotavirus: Measurement of Anti-Human Rotavirus Immunoglobulin G & M Levels by ELISA - Significant progress has been made in developing ELISA systems for the detection of antibody to the human rotavirus. The major difficulty which had to be overcome in adapting the ELISA for this purpose concerned the source of rotavirus antigen. Tissue culture grown virus, the usual source of antigen in solid phase antibody assays, was not available since the virus does not grow well in cell culture. While tissue culture grown antigenically related animal viruses could be used we did not find them to be efficient for detection of antibody. In addition, while the human viral antigen purified by density sedimentation could be used, this was not a practical approach. For this reason we investigated the possibility of using hyperimmune antirotavirus antibody attached to the solid-phase to capture unpurified rotaviral antigen from stool. Hyperimmune serum directed against rotavirus antigen was first adsorbed to the microtiter plate. A crude stool suspension derived from a gnotobiotic calf infected with human rotavirus was diluted in a non-ionic detergent (Tween) and an excess of a neutral protein such as fetal calf serum was then added. The only constituent of this suspension which was bound specifically was that which reacted with hyperimmune serum. The remainder of the material was removed by the washing procedure. The plates were then stored indefinitely at 4°C. The assay was performed by adding the serum or fluid to be tested. After a suitable incubation interval the plate was washed and appropriate enzyme labeled immunoglobulin was added. The excess conjugate was washed off and the substrate was then added. The substrate was changed from its colorless state to yellow

by bound enzyme and the reaction was proportional to the amount of immunoglobulin bound. Sera or secretions could be assayed for each subclass of antibody by using class specific antiserum.

This ELISA system proved to be four times as sensitive as the standard anti-human rotavirus fluorescent-antibody assay and ten times as sensitive as the standard anti-human rotavirus complement fixation antibody assay. In addition, the ELISA was capable of determining immunoglobulin G (IgG) and IgM subclasses of anti-rotavirus antibody using a single dilution of serum. With this assay, it was found that 11 of 21 infected children had anti-rotavirus IgM in their acute phase sera before the appearance of anti-rotavirus IgG.

The binding ELISA was also employed to study the anti-rotavirus secretory IgA (SCIgA) in 113 human milk and colostral specimens from a rural area in Guatemala, 32 colostral specimens from an urban area of Costa Rica and 12 from an urban area of the United States. Anti-rotavirus SCIgA was found in each colostrum specimen and in 94% of the milk examined. Both the absolute concentration of anti-rotavirus SCIgA and concentration relative to total SCIgA were highest in the colostra; the titer fell to lower but detectable levels in milk from 1 week to 2 years after birth. Significant differences were not noted for specimens from the three different locations.

Animal studies by others indicate that it is primarily local antibody which provides protection against rotavirus infection. Similarly, preliminary studies in our laboratory (described later) indicate that protection against rotavirus infection is afforded by anti-rotavirus SCIgA present in gastrointestinal fluids. It is thus likely that human colostrum and milk could protect the newborn against rotavirus infection by providing antirotavirus SCIgA when the ability of the gastrointestinal tract to synthesize this antibody is limited.

Measurement of Anti-Rotavirus Antibody by An Elisa Blocking Assay - As described above, the ELISA binding assay for the measurement of antibody to human rotavirus is highly efficient and sensitive. However, it requires antigen obtained from infected gnotobiotic animals, a reagent not widely available. In addition, a colorimeter is required for maximal efficiency. These disadvantages can be circumvented by an ELISA blocking assay which utilizes human rotavirus obtained from human stools and which can be read easily by the naked eye. As described above, this ELISA blocking assay was also employed to differentiate human and animal rotaviruses. It is analogous to a classical tissue culture neutralization assay in which the antigen and antibody are incubated together and the unbound antigen assayed subsequently. In the ELISA blocking test the antigen and antibody are incubated in a separate plate and then transferred to the microtiter plate precoated with hyperimmune goat anti-human rotavirus serum and the unneutralized virus is measured by the basic ELISA.

The ELISA blocking assay was equal in efficiency to the fluorescent antibody (FA) and ELISA binding tests for the detection of serologic responses to human rotavirus in infants and young children. However, the ELISA blocking and binding assays detected an antibody response in some adult contacts who did not have a response by FA. The FA assay is not as practical as ELISA as the requirement for microscopic examination of frozen sections of rotavirus infected animal intestine make it difficult to use in large scale studies. The ELISA binding assay has the advantage of being somewhat more sensitive and being able to measure immunoglobulin subclasses such as IgG and IgA but has the disadvantage of requiring either purified antigen or antigen derived from a gnotobiotic animal. In addition, the ELISA binding assay and the FA require a separate set of labelled reagents for each animal species tested while the ELISA blocking assay can be used to study the response of any animal infected with human rotavirus. The results of the ELISA blocking assay correlated well with those of the complement-fixation assay when sera from rotavirus positive children were studied. However, the CF assay was not efficient in detecting a seroresponse in their adult contacts.

While four different sources of human rotavirus were equally efficient as antigens for the ELISA blocking assay, NCDV was not as efficient. This lower efficiency of NCDV is similar to that found in the CF test. This result is also in keeping with findings discussed above which indicated that rotaviruses derived from different animal species could be distinguished by the ELISA blocking technique.

Thus, where the major need is the measurement of total rotavirus antibody, the simplicity and efficiency of the ELISA blocking technique and the more readily availability of antigens used in this assay suggest that this method may be readily applicable for use in the field as well as the laboratory.

Diarrhea Associated with Rotavirus in Rural Guatemala: A Longitudinal Study of 24 Infants and Young Children - A population of 24 infants and young children followed prospectively during the first three years of life in the Guatemalan highland village of Santa Maria Cauque was studied for the occurrence of rotavirus infection using enzyme-linked immunosorbent assay to detect virus in stools. Stool specimens were collected on a weekly basis from a cohort of 45 children for purposes of defining the pattern of intestinal colonization and of studying enteric illness. Specifically, for purposes of the current survey, 183 diarrheal episodes which occurred between 1964 and 1967 in 24 children of the original cohort of 45 were selected for the study. 557 stool samples, collected when possible before, during and after diarrhea were studied in sequence. Only diarrheal episodes occurring during the first 3 years of life were chosen for purposes of the present study and episodes associated with known enteric pathogens i.e., Shigella, Salmonella, enteropathogenic E. coli or acute parasitic infection were excluded. Infection with rotavirus was associated with 26 (14.2%) of 183 selected diarrheal episodes during the 3 study years. Twenty of the 24 infants and young children had

diarrhea associated with rotavirus on at least one occasion and 6 had 2 such episodes. The incidence of approximately 1.1 rotavirus diarrheal episodes per child during the first 3 years of life is an estimate based on selected diarrheal episodes excluding those associated with bacterial or parasitic pathogens (approximately 42%) and those from which stools were not available for study (approximately 5%). Although rotavirus was only associated with approximately 14% of selected diarrheal episodes in this survey, it accounted for a large proportion of severe diarrheal illness as defined by vomiting and dehydration. Rotavirus infection was associated with over 50% of the dehydrating diarrheal illnesses which occurred in this group of 24 children during the first 3 years of life. Also vomiting was 4 times more common among patients with rotavirus diarrhea compared to all other diarrheal episodes. This finding underscores the importance of diagnosis since if rotavirus is detected in specimens from a patient with diarrheal illness it is more likely that that patient will prove to have a dehydrating illness and careful attention must be paid to fluid and electrolyte balance. Thus, the application of immunologic assays to the study of these valuable specimens collected and carefully stored over 10 years ago has helped place rotavirus into perspective regarding its importance in traditional societies of the less developed countries.

Animal Models for Human Rotavirus Infection - Work with animal models is being pursued in order to assess virulence of rotavirus strains derived from man and animals as well as to define relationships between selected rotavirus strains based on their ability to cross-protect. Studies are underway using the "UK" strain of bovine rotavirus, which grows well in cell culture and has been plaque-purified, to assess the virulence of this strain in calves and piglets at Plum Island Animal Disease Center in collaboration with Dr. C.A. Mebus. In studies done in England, this strain of bovine rotavirus was highly virulent for calves and piglets. In contrast, Nebraska calf diarrhea virus (NCDV) appears to be less virulent in calves and does not produce diarrhea in piglets. We are pursuing studies with the "UK" strain to obtain clearance from the U.S. Department of Agriculture for further use of this virus in the U.S. The "UK" strain has been propagated and passaged serially in primary cell cultures and should meet initial criteria for a candidate vaccine strain. If it or another acceptable animal rotavirus could be shown to infect man without producing illness, it might be possible to immunize against human rotaviral disease in this manner.

The concept of cross-protection between heterologous strains of rotavirus from different species was evaluated in the newborn gnotobiotic calf. In sequential challenge studies (performed in collaboration with Dr. Mebus) initial infection in utero with bovine rotavirus induced complete resistance to disease when calves were challenged at birth with the human rotavirus. Thus these viruses appear to be sufficiently related for the bovine virus to induce immunity to the human rotavirus.

In Vitro Cultivation of Rotaviruses - While the human rotavirus has not been grown to a high titer in tissue culture, several animal rotaviruses have been cultivated successfully. The "UK" strain and NCDV represent two such bovine rotaviruses. Other strains include a calf rotavirus from Northern Ireland, simian rotavirus (SA-11) from South Africa, and "O" agent from intestinal washings of sheep and cattle from South Africa. Recently, it was reported that cultivation of a Canadian calf rotavirus and a U.S. pig rotavirus was significantly enhanced by incubating trypsin, chymotrypsin, or pancreatin with virus prior to inoculation of tissue culture. We have confirmed this effect and have seen a 10-32 fold increase in infectivity, as measured by plaque assay, following exposure of bovine rotavirus to trypsin. Unfortunately, the optimal conditions of trypsin enhancement that increased infectivity of the bovine rotavirus did not have a similar effect upon the human rotavirus. To date, various concentrations of proteases have not induced the human rotavirus to grow efficiently in tissue culture.

Two strains of animal rotavirus, previously adapted to cell culture, were studied for plaque-forming ability in a variety of cell types. The "UK" bovine rotavirus produced plaques in primary African green monkey kidney (AGMK), primary calf kidney, and in two continuous cell lines (CV1 and MA104); AGMK cells were preferable because of consistency in plaque production. The SA-11 rotavirus also produced plaques in AGMK cells. Neither virus required the addition of proteases or other enzymes for plaque production, however, as mentioned above, infectivity of the bovine virus was increased 10-32 fold by trypsin treatment. Both rotaviruses were subsequently plaque-purified in AGMK cells.

The ability of the UK bovine virus to produce plaques at 32°, 37°, 38°, 39° and 40°C was studied. Plaquing efficiency was poor at 32°, but conditions appeared to be optimal at 38°, which was the temperature chosen for further study. Well-defined plaques were seen at 39° and 40°C indicating that the wild type "UK" virus was not temperature sensitive.

Study of Rotavirus Infection in Adult Volunteers - A safety tested inoculum consisting of a dilution of a 2% stool filtrate prepared from a stool rich in rotavirus particles obtained from a 13 month old child hospitalized with gastroenteritis has been administered orally to 18 volunteers once and to 2 of these volunteers a second time, 19 months after the initial challenge. These studies were performed in 6 phases. The first was carried out at the Clinical Center, NIH and the remainder at the University of Maryland Center for Vaccine Development in Baltimore, Md. In initial studies, volunteers with a high level of serum rotavirus antibody were selected, whereas later, when feasible, individuals with the lowest available antibody levels were challenged. Four of the 18 volunteers developed a diarrheal illness on initial challenge whereas 2 developed a febrile illness without diarrhea or vomiting. Each of the 4 volunteers who developed diarrhea shed rotavirus in stool. Preliminary study of jejunal fluid of 9 volunteers for IgA antibody levels by ELISA

revealed that the presence of IgA antibody in jejunal fluid may be the key determinant to susceptibility or resistance to the development of rotavirus illness. Additional fluids are available on 4 of the initially challenged and 2 of the rechallenged volunteers from the most recent study and these will be studied for IgA antibody responses to rotavirus by ELISA shortly. Two of the volunteers who were rechallenged developed a diarrheal illness on initial challenge 19 months previously. On second challenge, one remained well, whereas the other developed mild signs and symptoms which included nausea and malaise but not diarrhea or vomiting; he did not shed rotavirus. The sequential jejunal fluids obtained prior to and after each challenge from these 2 volunteers will be evaluated for IgA antibody responses to rotavirus by ELISA. In summary, following initial challenge 4 of 18 volunteers developed gastroenteritis and 2 had a febrile illness without diarrhea. Although not all serologic studies are completed, it appears that individuals with a low level of pre-existing serum antibody became infected and developed illness more often than persons with a high titer of serum antibody. Thus, we appear to have identified a serologic correlate of resistance to the virus. However, it appears from preliminary studies that local intestinal immunity secretory antibody plays a more important role than serum antibody in resistance. It has been difficult, to identify adults without pre-existing serum antibody to the human rotavirus. The effort has been worthwhile, however, since it is now clear that illness can be induced in some adults who have low levels of serum antibody. This finding has implications for the development of effective immunoprophylaxis since the safety and efficacy of potential live vaccines can be evaluated in such susceptible adult volunteers. Also the mechanism of immunity to human rotavirus is amenable to study in such individuals.

Characterization of Rotavirus RNA and Comparison of Rotavirus Strains by Gel Electrophoresis of Viral RNA - Comparison of the RNA patterns of animal and human rotavirus RNA's were made. Animal viruses studied included the NCDV and UK calf rotaviruses, the "O" agent and SA-11 and two porcine rotaviruses, OSU and EE. Two isolates of human rotavirus from each of four winter seasons (1974-1976) were passaged in gnotobiotic calves and the virus from calf feces was purified and used for RNA analysis by gel electrophoresis.

Coelectrophoresis comparisons of the human strains revealed 3 different patterns which formed the basis for grouping them. Group I and Group II differed in the migration of segment no. 5. Groups I and II differed from Group III in the migration of segments no. 6, 9, and 10. Comparisons among the animal strains resulted in a pattern with differences in the migration of 3 or 4 segments usually in the largest sized RNA class. Comparisons between the porcine rotavirus strains and other animal rotavirus strains frequently produced a difference in the migration of the 7th segment. When the RNA's from the human and animal strains were coelectrophoresed differences in the migration of 5 or 6 segments resulted and these differences were in all 4 size classes of RNA. In comparisons

with the human and animal strains, frequent differences were observed in the migration of the 3rd, 4th, 5th, 9th and 10th segments. Gel electrophoresis of RNA is a sensitive technique for identifying rotaviruses following in vivo and in vitro propagation and may prove useful for studying natural transmission of rotaviruses.

Gel electrophoresis of viral RNA will also be of value in genetic studies with rotaviruses. The ability to distinguish each RNA segment of one rotavirus strain from the corresponding segment of a second strain is essential for genotyping. Work is proceeding on the use of a discontinuous gel electrophoresis method which has been practical for genetic studies with reoviruses, and this may offer the same advantage with rotaviruses. This system may provide added resolution over our currently employed system.

Rotaviral Proteins - The simian rotavirus (SA-11) was chosen as a model for study of rotavirus polypeptides since it grows well in cell culture and possesses a hemagglutinin associated with its outer capsid. Two peaks from a bouyant density fractionation of cell-culture-grown SA11 virus were subjected to gel electrophoresis. The first peak with a buoyant density of 1.403 g/cm^3 in CsCl contained about 70% single capsid particles, and was RIA positive and HA negative. The core particles in this peak yielded 5 polypeptide bands on a phosphate buffered polyacrylamide gel. The second peak with a bouyant density of 1.370 g/cm^3 in CsCl contained approximately 90% double capsid particles and was RIA and HA positive. The virus in this peak was fractionated into 8 polypeptide bands which ranged in size from 52,000 to 118,000 daltons. Three of the polypeptides detected in this second peak are assumed to be located in the outer capsid of the simian rotavirus. The phosphate buffered continuous gel system employed in this experiment appears to be comparable to the more commonly employed discontinuous tris-glycine gel systems. It would appear that resolution of fewer polypeptides reported by others using continuous gel systems was due more to a low concentration of double capsid virus in the starting preparation than to the gel system employed.

Further characterization of rotavirus polypeptides is proceeding with the use of ^{35}S-methionine incorporation during growth in vitro. Labeled virus which is fractionated on discontinuous tris-glycine gels will be subjected to autoradiography. When cytoplasmic extracts are prepared the total number of viral polypeptides produced during rotavirus replication will be determined. Use of this technique is planned for those rotaviruses which are adapted to growth in cell culture. Despite inability of the human rotavirus to grow efficiently in cell culture, incorporation of ^{35}S-methionine during in vitro replication of human rotavirus appears possible. A recently described technique which employs low speed centrifugation to enhance virus uptake and permit single cycle replication in these cultures, encourages us in the use of gel electrophoresis and autoradiography with the human rotaviruses. If successful, such experiments may allow us to determine which polypeptides are not produced

in sufficient quantity to permit efficient growth of human rotavirus in vitro. Work is also proceeding on a method of preparation of antiserum to each of the individual rotavirus structural polypeptides by using gel electrophoresis to fractionate purified virus into its component antigens.

Hemagglutination of Rotavirus - The simian rotavirus (SA-11) was shown to hemagglutinate human group "O" and guinea pig erythrocytes, and hemagglutination was inhibited by specific hyperimmune anti-SA-11 guinea pig serum. The hemagglutinin appeared to be associated with the outer capsid of the SA-11 virion since fractionation of SA-11 infected cell culture fluid on a CsCl density gradient produced an HA positive peak with a density of 1.359 g/cm^3 and 90% of the virus particles in this peak had the outer capsid present.

Cell culture grown NCDV, UK calf, "O" agent, OSU porcine rotaviruses and fecal preparations of human rotaviruses from gnotobiotic calves are being investigated for their ability to agglutinate erythrocytes from man (group "O") and a variety of animals (rat, monkey, guinea pig, cow and chicken) at differing temperatures. Since type-specific antigens may reside in the outer capsid, the HA of rotaviruses may prove useful in defining rotavirus types. In addition HA provides a biological marker which will be of great value in genetic studies with rotaviruses. Future experiments are planned to determine which vival polypeptide is responsible for HA activity and to make antiserum to the specific HA polypeptide. Additional effort is planned to confirm the detection of a human rotavirus hemagglutinin recently reported by Japanese investigators.

Enhancement of Antigen Incorporation and Infection of Cell Culture by Human Rotavirus - Work was begun to study the antigenic relationships of rotavirus serotypes using a test described in the United Kingdom. This technique, described by Flewett and co-workers, involves the low-speed centrifugation of stool suspensions onto monolayers of monkey kidney cells and the subsequent demonstration of infection by immunofluorescence. Antigenic characterization is then performed by cross-neutralization. Initial attempts at performing the neutralization test were frustrated by our inability to standardize the antigen dose because of the great variability in infectivity from test to test. Growth-curve studies at this stage failed to show evidence of replication by any of the human rotavirus strains tested. Subsequent work was then concerned with the determination of factors involved in infectivity of tissue culture monolayers by human rotavirus.

Three factors were found to be of importance in infectivity of human rotavirus for tissue culture. Firstly, aggregation of virus particles appears to be a prerequisite to infection. Mild sonication (insufficient in itself to reduce the titer of infectious virus) followed by filtration through a 450 nm filter (which did not result in any loss of total antigenicity as determined by radioimmunoassay) caused a precipitous drop in infectivity as compared to untreated specimens. In contrast, a decrease in infectivity with the tissue culture-adapted simian rotavirus

(SA-11) was not seen and only a mild drop of infectivity with the in-vitro cultivable calf rotavirus was noted when these viruses were treated in a similar way. Secondly, low-speed centrifugation was found to be an important factor in infectivity. In the absence of centrifugation, human rotavirus failed to infect monolayers, while infectivity of the calf rotavirus was markedly diminished by the absence of centrifugation and that of SA 11 only marginally diminished. The minimal effective centrifugation time was found to be about 20 minutes. Thirdly, incorporation of trypsin in the inoculum to a final concentration of 10 ug/ml enhances infectivity of the human strains, but not the calf or simian strains. This enhancement of infectivity appears to be an effect on the virus rather than on the tissue culture cells, as pre-treatment of cells did not result in increased infectivity, whereas pre-treatment of virus did.

By means of immunofluorescence, distinct membrane fluorescence could be observed in cells infected with human strains immediately after centrifugation and granular cytoplasmic fluorescence was observed from 6 to 12 hours after infection, which slowly progressed in intensity of fluorescence and number of cells infected. In contrast, the non-human strains inoculated at doses which produced little or no membrane fluorescence showed bright intracytoplasmic fluorescence at 6 hours, rapidly increasing to involve most cells in the monolayer. Thus, there appears to be a dichotomy with respect to conditions required for tissue culture infectivity between human rotavirus strains and the non-human strains which can be propagated in tissue culture.

Serial passage of monolayers infected by human virus under conditions described above revealed that viral antigen was detectable by immunofluorescence and ELISA up to the third passage, although the percentage of infected cells decreased considerably with each passage.

Infectivity of cell cultures and in vitro human rotavirus protein formation was demonstrated by ^{35}S methionine incorporation, and the specificity of this de-novo synthesized viral material was established by radio-immunoprecipitation.

Role of Norwalk Virus in Outbreaks of Non-Bacterial Gastroenteritis - The development of a radioimmunoassay (RIA) for detection of Norwalk antigen and a radioimmunoassay blocking (RIABL) test for detection of antibody to the Norwalk agent as well as the development of an immune adherence hemagglutination assay (IAHA) for detection of purified high titered Norwalk antigen and of Norwalk antibody were described in last year's annual report. The RIA and RIABL are more sensitive than the IAHA and employ less antigen than IAHA and thus have become a mainstay in studying the epidemiology of Norwalk virus infection. The availability of the RIA and RIABL have permitted large scale epidemiologic studies of Norwalk infection. Before the development of the serologic assays noted above IEM was the only method available to study Norwalk infection but was impractical for large scale seroepidemiology.

Important progress was made in studying the natural history of Norwalk infection by examining specimens from numerous outbreaks of gastroenteritis. Acute and convalescent phase sera from a total of 25 separate gastroenteritis outbreaks had been gathered by us and other investigators over the past twelve years. In most outbreaks the well established causes for epidemic diarrhea such as Shigella and Salmonella had been excluded. The outbreaks occurred in a variety of locations including cruise ships, Naval vessels, schools, nursing homes, communities and hospitals. The minimum number of paired sera available from an outbreak was 1 and the maximum 25 with an average of 9.4. Sera were stored at -20°C until tested. Paired sera were collected at the time of acute disease and during convalescence. The interval between acute and convalescent sera varied from outbreak to outbreak with an average of about 1 month. The shortest interval was 2 weeks and the longest 8 weeks. In two outbreaks acute phase stool specimens were available and examined by RIA for the presence of Norwalk antigen.

Six (26%) of the 23 newly studied outbreaks appeared to be related to the Norwalk virus. In five outbreaks (2 in different colleges, 2 on cruise ships and one in a community in Japan) greater than 50% of the individuals tested showed a four fold or greater antibody rise. In the sixth outbreak which originated in a school and involved the study family only one set of paired sera was available and in this instance a seroresponse was detected. In addition Norwalk antigen was detected in this patient's stool during the acute illness. Norwalk antigen was also detected in selected stools from one of the cruise ship outbreaks (Pacific area). Actually, this outbreak was a series of outbreaks of gastrointestinal illness on a cruise ship which occurred during successive cruises. Affected individuals from the 1st cruise developed a significant antibody rise, while stools from the 1st and 3rd cruises were positive for the Norwalk virus by RIA. The only other cruise ship outbreak tested also appeared to be Norwalk related. Interestingly, this cruise ship (Carribbean area) also had a series of outbreaks on successive voyages. Serologic evidence of Norwalk infection was detected during the third outbreak, unfortunately the earlier outbreaks were not studied. In two previously described outbreaks, the original Norwalk, Ohio epidemic and the Montgomery Co. outbreak, an association with the Norwalk virus had been demonstrated by studies in volunteers as well as by IEM study of paired sera derived from each outbreak. In an additional three outbreaks a small proportion of individuals had a seroresponse to the Norwalk agent. These rises, while significant, tended to be of lesser magnitude and occurred less frequently than in the outbreaks described above.

The outbreaks were also investigated for serologic evidence of infection with human rotavirus. While this virus appears to be predominantly a pediatric pathogen, there is increasing evidence it can cause illness in adults. In one outbreak aboard a naval vessel each of 3 individuals tested developed a seroresponse to rotavirus. It is of interest that in this outbreak the seroresponses were directed to the type 1 rotavirus

rather than the more common type 2 virus. Representative serum pairs from each of the newly studied outbreaks were tested for a serologic response to Escherichia coli heat-labile enterotoxin (LT) using a solid phase blocking radioimmunoassay. In no instance was a serologic response to LT encountered.

Thus, in this preliminary survey Norwalk virus or a Norwalk related virus appeared to account for a substantial percentage (26%) of epidemic acute infectious non-bacterial gastroenteritis. Also, the serologic evidence of infection on two cruise ships and in Japan confirms our previous finding that Norwalk-like infection is not limited to the United States. (We had found that by IAHA that Norwalk antibody was also present in Bangladesh.) If one includes the original Norwalk outbreak as well as the serologically related Montgomery County outbreak, 8 separate outbreaks of Norwalk gastroenteritis were identified among 25 outbreaks studied (32%). It is possible that a majority of undiagnosed non-bacterial gastroenteritis might have an assignable etiology if satisfactory serologic assays existed for the antigenically distinct 27 nm Hawaii and "W" viruses which share biophysical and epidemiologic properties with the Norwalk virus.

Other 27 nm Gastrointestinal Viruses - We have also been closely involved in collaborative studies with investigators in different parts of the world who have described small Norwalk-like particles in feces of patients with gastroenteritis. We are functioning in an informal sense as a reference laboratory to sort out these agents and to study their relationships to our 27 nm particles. One group of agents from England of approximate Norwalk size, but described as astrovirus because the particles resemble star-like structures by negative staining (Kurtz et al., J. Clin. Path., 1977), has been examined for its relationship to the Norwalk agent by IEM and by RIA and none was found. Significantly in our tests, these viruses appear to be indistinguishable from the Norwalk virus by negative staining. We have also received specimens which had been described as containing particles of approximate Norwalk size from Canada, and have just received similar specimens from Australia and are awaiting similar specimens from Japan.

ELISA for Detection of Adenoviruses - Another group receiving renewed attention is the adenoviruses. In the Children's Hospital study cited earlier, adenoviruses were the second most commonly detected virus in stools of patients with gastroenteritis; in the initial study 16 of the 143 patients shed adenovirus in their stools. Of interest, only 6 of the 16 adenoviruses were detected by routine tissue culture methods, whereas 14 of the 16 were detected by electron microscopic examination of stool filtrates or rectal swab specimens. Further efforts to isolate EM positive, and tissue culture negative adenoviruses in tissue culture were made and a few additional strains were recovered; however, several were not successfully recovered indicating that there are fastidious adenoviruses which can be visualized by EM but which are not readily detectable in tissue culture. However, in serologic (CF) studies only

3.6% of the patients with diarrhea had evidence of adenovirus infection, a frequency similar to that observed in the control group which consisted primarily of patients with respiratory tract disease. Since the frequency of an adenovirus seroresponse was similar in the diarrhea and control groups, adenovirus infection could not be associated with diarrheal illness. This finding does not exclude a contribution of these viruses to enteric disease equivalent to that known to occur in pediatric respiratory tract illnesses. With the information from this study, and data from studies of others which had associated adenoviruses with gastroenteritis, we felt that the role of these agents should not be ignored and thus a simple detection test for adenovirus in stools similar to the ELISA for rotaviruses was needed. We have developed an assay for detection of adenovirus in stool preparations using an indirect ELISA in which an adenovirus hyperimmune rabbit serum is utilized as precoat, an adenovirus hyperimmune guinea pig serum as the second antibody, and a goat anti-guinea pig serum conjugated with alkaline phosphatase as the third antibody. The efficiency and sensitivity of this system for detecting adenoviruses in stool material is currently being evaluated. We plan to continue to utilize this adenovirus ELISA to screen specimens from various studies in an attempt to determine the role, if any, of adenoviruses in acute infectious, non-bacterial gastroenteritis. A binding and blocking ELISA for antibody for adenovirus appears to be working satisfactorily but needs further evaluation for its sensitivity and efficiency.

Coronaviruses - We are also attempting to evaluate the role of coronaviruses in acute gastroenteritis of man. Other investigators have associated these agents in a preliminary fashion with outbreaks of gastroenteritis in adults, but the role of coronaviruses is still uncertain since they have not been observed with certainty in infantile gastroenteritis and their role in adult illness needs confirmation in other laboratories. Since coronaviruses do cause severe gastroenteritis in piglets, calves, dogs and turkeys, attempts to elucidate the role, of these viruses, if any, in human gastrointestinal disease must be pursued. We have not yet visualized definite coronavirus particles in any stool specimen examined by EM. We have received the calf coronavirus from Dr. Mebus and inoculated it into suckling mice for preparation of HA and CF antigens. We have tested paired sera from hospitalized rotavirus-negative gastroenteritis patients to determine if serologic responses could be detected to the calf coronavirus since it was felt that this agent might cross-react with a putative human enteric coronavirus, a relationship which might be analogous to that between the human and animal rotaviruses. However, seroresponses were not observed to the calf rotavirus antigens. Obviously, attempts at elucidating the role of coronaviruses in human enteric diseases will be an important priority item in the laboratory.

Enterotoxigenic Escherichia coli Diarrhea of Traveler's: A Prospective Study of American Peace Corps Volunteers - We collaborated in a study with Dr. David Sack of Johns Hopkins University and other colleagues. Travelers' diarrhea was studied prospectively in a group of 39 American Peace Corps Volunteers (PCV's) during their first five weeks in Kenya.

Twenty-seven developed diarrheal disease and 12 remained well. Multiple episodes were documented in 11 of the symptomatic volunteers. Enterotoxigenic E. coli of many serotypes producing heat-labile and/or heat-stable enterotoxin were isolated from 17 of the 27 volunteers with diarrhea and from 1 of the 12 well volunteers. The enterotoxigenic E. coli were more often antibiotic sensitive than the non-enterotoxigenic E. coli. A serum antibody rise to the heat-labile toxin (LT) was detected in 6 symptomatic volunteers, 5 of whom had a positive culture for LT-producing E. coli, and from one asymptomatic, culture negative volunteer. Salmonella cubana was isolated from two volunteers, and three volunteers had serologic evidence of infection with human rotavirus. This study confirms the role of enterotoxigenic E. coli as a major cause of travelers' diarrhea and suggests that the disease is similar in widely separated geographic areas.

Prophylactic Doxycline for Travelers' Diarrhea - In another collaborative study with Dr. David Sack of Johns Hopkins University LID performed the serologic (CF) studies for rotavirus antibody on paired sera. A randomized double-blind study to determine the efficacy of doxycycline (100 mg daily) in preventing travelers' diarrhea among 39 Peace Corps volunteers during their first five weeks in Kenya was performed. The volunteers took either doxycycline or placebo for three weeks and were observed for an additional two weeks. Nine of 21 taking placebo and one of 18 taking doxycycline had travelers' diarrhea during the treatment period ($P = 0.012$). The protection seemed to persist for at least one week after the drug was stopped. Enterotoxigenic E. coli was the only pathogen isolated from the placebo group, but was not detected in persons taking doxycycline. None of these organisms was resistant to doxycycline or tetracycline, whereas resistance to tetracyclines and other antibiotics was common among the nonenterotoxigenic E. coli. Paired sera from 37 volunteers were tested for a serologic response to rotavirus; only one individual, an asymptomatic member of the doxycycline group had a 4-fold rise in titer to rotavirus. We concluded that doxycycline effectively prevented most episodes of travelers' diarrhea.

Enzyme-linked Immunosorbent Assay (ELISA) for Detection of Escherichia Coli Heat-Labile Enterotoxin - An enzyme-linked immunosorbent assay (ELISA) for the detection of heat-labile E. coli enterotoxin was developed. The assay, which is based on the immunological similarity between Vibrio cholerae toxin and heat-labile E. coli enterotoxin is similar in design to a radioimmunoassay but utilizes enzyme-labeled rather than radioactive isotope-labeled reagents. The ELISA system is as sensitive as both radioimmunoassay and the Y-1 adrenal cell assay for the detection of heat-labile E. coli enterotoxin but requires neither radioactive reagents nor tissue culture techniques. The ELISA is easy to perform and is adaptable for use in small laboratories.

Inflammatory Bowel Disease and Gastrointestinal Viruses - We were also involved in the search for etiologic agents of chronic inflammatory bowel disease. Initially this was carried out in a rather limited and sporadic fashion. However, with a report last summer by University of Vermont investigators that they had isolated an agent from Crohn's disease tissue which was an RNA virus, 55-60 nm in diameter, heat, ether and acid stable and antigenically related to the NCDV rotavirus, our efforts in this area were intensified. This was especially so since a volunteer study in which we were planning to administer the human rotavirus once again, had to be cancelled because of the report. For this reason we undertook an intensive investigation of some of the Vermont material in order to determine if rotavirus was indeed present. Using tissue culture, ELISA immunofluorscence and to a lesser extent, IEM or combinations of these techniques, we were unable to demonstrate the presence of rotavirus in the Vermont material which was studied intensively. However, in collaboration with Dr. Barile (BOB) and Dr. Tully, fastidious mycoplasma organisms were detected. We have collaborated with the University of Vermont investigators in clarifying the contents of their material. In addition, we are collaborating in other studies with them in an attempt to determine seroepidemiologically whether Crohn's disease patients have differences in prevalence of antibody to rotavirus and Norwalk virus.

In another investigation we studied the prevalence and titers of antibody to the Norwalk agent and rotavirus in Crohn's disease and control patients. All patients and controls were residents of the Minneapolis, Minnesota area. One group of 37 Crohn's disease patients, primarily males, was studied. They ranged in age from 16 to 53 years, were outpatients, and their illness were in various stages of clinical activity at the time of study. The diagnosis of Crohn's disease was made by one of three clinical gastroenterologists using established criteria including clinical course and pathologic and/or radiologic examinations. The Crohn's disease group was compared with 2 groups of controls. One control group consisted of 30 normal hospital employees (physicians and technicians) who were compared with 30 of the 37 Crohn's patients. The other control group was made up of 37 hospitalized patients with various diseases, primarily cirrhosis who were compared with the 37 Crohn's patients. The controls were matched to the patients with Crohn's disease on the basis of age, sex and time of serum sampling which differed by no more than 1 month. No significant differences in prevalence of geometric mean titers of rotavirus or Norwalk virus antibody were observed between test and control groups.

Diarrhea in a Prospective Study of an Indigenous Adult African Population - We collaborated in a study with Dr. Hansen who was at the University of Nairobi and other colleagues by performing serologic (CF) studies using rotavirus (Mr. L. Cline). A prospective study of 77 indigenous African adults with acute diarrhea seeking care at the major hospital in Nairobi, Kenya, to determine the major pathogens responsible for this syndrome in adults was conducted. Fecal and blood specimens were collected and

examined for enteric bacterial pathogens, viruses, and parasites. In 13 (26%) inpatients and 11 (49%) outpatients Shigella was found, and heat-labile and heat-stable forms of enterotoxigenic Escherichia coli were found in 9 (18%) inpatients and 1 (4%) outpatient. Human reovirus-like agent titers rose significantly in another 3 (6%). Amebic dysentery was not seen although hemagglutination-inhibition tests for invasive Entamoeba histolytica were postive in 4 inpatients. An etiologic agent was found in 65% of patients.

Significance to Biomedical Research and the Program of the Institute

Acute infectious nonbacterial gastroenteritis is a common infectious disease which affects a broad segment of the population and was the second most common disease observed in a 10 year family study in the United States. In addition, diarrheal diseases are a leading cause of mortality as well as morbidity in developing countries. Ultimate goals of prevention and therapy have been furthered in our current studies by (1) the detection and preliminary characterization of etiologic agents of this disease, (2) the development of sensitive, efficient and rapid assay systems by which the epidemiologic importance of the disease can be determined, (3) the in vitro, although limited, cultivation of the human rotavirus, (4) the ability to induce illness in human volunteers and experimental animals administered the human rotavirus (5) the ability to differentiate the human rotavirus from other related agents by biophysical and serologic methods and (6) the elucidation of the epidemiology of the rotavirus and Norwalk viruses. The progress in studying the role of the human rotavirus the etiology of gastroenteritis in infants and young children represents a significant step in understanding this disease of worldwide importance. In addition, the development of sensitive assay systems for the heat labile toxin of E. coli should aid in elucidating the role of toxin producing E. coli during epidemiologic studies.

Proposed Course of Project

In future studies of the Epidemiology Section major emphasis will be given to the efficient propagation of the viral gastroenteritis agents in vitro, to the delineation of their overall importance over a sustained period in the etiology of gastroenteritis in various populations, to the immune mechanisms involved in host-defense, to the development of effective methods to prevent illness due to these agents, and to the continued search for other etiologic agents of acute infectious gastroenteritis.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00022-20 LID

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

STUDY OF NONBACTERIAL RESPIRATORY DISEASES IN CHILDREN

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert M. Chanock LID/NIAID Chief

COOPERATING UNITS (if any)

Children's Hospital National Medical Center, Washington, D.C.

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

No work has been done on this project during the past year.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00024-18 LID

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
LABORATORY STUDIES OF MYXOVIRUSES

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Respiratory Viruses Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
150/12	78/12	72/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Influenza A virus ts mutants and recombinants were evaluated in vitro and in experimental animals for evidence of growth restriction and genetic stability. Antigenicity and protective effective were also studied in vivo. New ts mutants were produced and characterized genetically. These and the vaccine candidate ts-1[E] and ts-1A2 recombinants are being genotyped by polyacrylamide gel electrophoresis. The ts genes in the A/Udorn/72 ts-1A2 recombinants were shown to effect a predictable level of attenuation when transferred into recombinants bearing the surface antigens of A/Victoria/75(H3N2), A/Alaska/77(H3N2) and A/Hong Kong/77(H1N1). Thus, influenza viruses belonging to different subtypes can be satisfactorily attenuated by the acquisition of the ts-1A2 mutations. Furthermore, the resulting ts-1A2 recombinants exhibit a remarkable degree of genetic stability in vivo.

Project Description

Objectives - (1) To produce conditional lethal temperature sensitive (ts) mutants of myxoviruses for possible use in live attenuated vaccines; (2) to study the genetic basis for attenuation shown by temperature sensitive mutants; (3) to define the relative importance of the various host defense mechanisms in resistance to myxovirus infection and disease.

Background

The explosion of new information concerning the structure and function of the influenza virus genome provides a basis for deliberate manipulation of its genes with the intent of introducing specific mutations into the virus that render it attenuated and potentially useful for prevention of disease. Viruses bearing specific, identifiable mutations that are responsible for attenuation represent the live vaccine strains of the future since the genetic basis of attenuation can be monitored directly during all phases of vaccine development, manufacture and utilization in man. Antigenic variability of influenza virus does not contraindicate the use of defined, identifiable, attenuating mutations for generation of live vaccine strains. On the contrary, this approach offers the only hope for controlling influenza epidemics or pandemics by live virus vaccine. The interval between recognition of a new, potentially epidemic or pandemic virus and its spread throughout the world is rather short, perhaps 5 to 6 months. It is essential, therefore, that at the time of an epidemic or pandemic threat live vaccine strains be developed and evaluated rapidly. Attenuation can be accomplished quickly by transferring mutant genes from an attenuated master strain to the new epidemic or pandemic strain by genetic reassortment. It is implicit in this approach that the mutations responsible for attenuation not affect the genes that code for the hemagglutinin and neuraminidase surface glycoproteins, otherwise these mutations could not be transferred to recombinants bearing the surface antigens of the new epidemic or pandemic virus. It is also implicit that the genetic lesions present in a recombinant influenza virus must be identifiable by simple in vitro techniques.

Major Findings

Isolation and characterization of temperature-sensitive mutants of influenza A virus - ts mutants were studied in greater detail because of the accumulating evidence that ts mutations are capable of attenuating influenza A virus and that they can be identified by simple in vitro techniques. We have isolated new ts mutants derived from a plaque-purified, virulent clone of A/Udorn/72 wild type virus grown in the presence of 5-fluorouracil. More than 1100 clones were evaluated and 16 were ts. Two of these had a 37°C shutoff temperature for plaque formation in tissue culture. Thirteen shutoff at 38°C and one at 39°C. Complementation tests with 12 of the new ts mutants and the 7 complementation groups represented by the HK mutants revealed that 3 of the new mutants fell into two new groups that complemented each of the standard 7 HK ts

probes. The 12 Udorn/72 mutants were also tested in hamsters for genetic stability. Three of them, ts-368 (HK group 2), ts-651 (HK group 6), and ts-559 (new "group 8"), exhibited an in vivo "reversion" frequency lower than that of Udorn/72-ts-1[E] virus, which bears two ts lesions and was used as a control. It appears that the 3 mutants possess characteristics desirable for a single ts lesion mutant donor virus. In addition, we have expanded our number of ts probes for complementation analysis to a total of eight (six distinct HK groups plus two new Udorn/72 groups). By analogy with the results of complementation tests between the HK mutants and the WSN group of mutants isolated by Sugiura and others and with the results of polyacrylamide gel electrophoresis performed by Palese and Ritchey with RNA extracted from the WSN mutants and our HK mutants, it is likely that ts-368 has a mutation in the gene coding for ribonucleoprotein (RNP) and that ts-651 has a mutation in the P2 (polymerase) gene. Both of these genes are necessary for virion RNA (vRNA) synthesis. A double mutant derived from these two strains would be analogous to ts-1A2, which has its two defects in genes necessary for cRNA synthesis (P3, P1).

The HK/68-ts-1[E] master vaccine strain bears its ts lesions on the P3 (polymerase) gene required for cRNA synthesis and on the RNP (ribonucleoprotein) gene necessary for vRNA synthesis. Single lesion ts mutants segregated from the ts-1[E] strain are unstable in vivo. The reversion frequency to ts^+ phenotype in the hamster lung is greater than 50% for each. We now have available single ts mutants with their lesion in the P3 gene (derived by segregation from the Vic/75-ts-1A2 virus) or the RNP gene (Udorn/72-ts-368) which exhibit greater genetic stability (less than 20% reversion frequency). It is likely that a double ts mutant derived by combining these single mutations would be more defective and stable in animals and man than the original ts-1[E] virus. This type of double recombinant will be prepared since there may be a need for recombinants less defective than ts-1A2 recombinants for use in individuals with pre-existing neuraminidase immunity.

Additional ts mutants of influenza A virus were produced by using mutagens that are known to cause deletion mutations in bacterial systems. It was hoped that a deletion mutation at the end of one of the segments of influenza A genome might yield a mutant that is both ts and genetically stable.

Influenza A/Udorn/72 (H3N2) virus was mutagenized with ICR 191 (an acridine-based compound), nitrous acid, or ultraviolet light and 6000 clonal populations were examined. 135 clones were temperature-sensitive for plaque formation on MDCK (canine kidney cells), i.e., plaque titer $40°/34° \leq 10^{-4}$. 85 of these 135 clones were also ts on rhesus monkey kidney (RMK) cells. Thus, 50 clones were less ts on RMK cells than on MDCK cells. 22 of these 50 clones were not ts on RMK cells, i.e., plaque titer $40°C/34°C > 10^{-2}$. These 22 mutants appeared to be temperature dependent, host range (td-hr) mutants. To determine if the td-hr mutation was present on one or more genes, complementation-recombination assay

was performed at 40°C on MDCK cells. These mutants did not share a common td-hr lesion, but instead fell into 8 groups indicating that a mutation affecting any of the genes represented by 8 different complementation groups resulted in host dependent restriction of plaque formation at high temperature. This further suggested that each influenza gene can undergo host range mutation.

The 85 mutants that were ts on RMK cells fell into 12 complementation groups when tested on these cells. More than 90% of the progeny from pairwise crosses involving these 12 groups yielded wild type (ts^+) virus except in the following crosses: 1 X 11, 3 X 11, 5 X 7, 5 X 9, 5 X 12 and 7 X 9. Progeny from 5 X 7, 5 X 9 and 7 X 9 were predominantly (>90%)ts virus suggesting that these 3 complementation groups represented only one recombination group. Complementation between groups 5, 7 and 9 probably represents intrasegmental or intracistronic complementation. Progeny from the 1 X 11, 3 X 11, 5 X 12, 7 X 12, and 9 X 12 crosses were mixtures of approximately equal numbers of ts and ts^+ viruses. This suggests that the prototype mutant of complementation group 12 has a ts lesion belonging to 5,7,9 recombination group. Also the prototype of complementation group 11 appears to have discrete ts lesions belonging to 2 separate recombination groups, i.e., group 1 and group 3. In other words, the complementation group 11 mutant appears to be double mutant. This hypothesis will be tested by mating these viruses with an antigenically distinct wild type virus and searching for single lesion ts segregants. In summary our Udorn/72 ts mutants can be arranged into 8 non-overlapping recombination groups. This is in good agreement with the currently accepted value of 8 influenza A genes.

To determine the genetic locus of the ts lesion in complementation groups 5 and 9, the Udorn/72 ts mutants representing these groups were mated with a WSN-ts mutant and ts^+ progeny viruses were isolated. Preliminary evaluation of ts parental and ts^+ recombinant viruses by polyacrylamide gel analysis suggests that both of the complementation group 5 and 9 Udorn/72 ts mutants have a ts defect on RNA segment 8, believed to code for the NS protein. This result is consistent with the interpretation that both mutants belong to the same recombination group. If this is confirmed, it would support the suggestion that the genetic interaction seen with groups 5 and 9 is indeed intracistronic complementation. These mutants appear to be among the first single ts mutants with defects localized to the NS gene.

The level of temperature sensitivity of the 135 mutants that were ts on MDCK was determined. Three had a 37°C shutoff temperature, 15 had a 38°C shutoff, 53 had a 39°C shutoff, and 64 had a 40°C shutoff. These 135 mutants fell into 8 complementation groups when tested on MDCK tissue. Further study of those mutants that have potential for use as vaccine strains (or as a donor of a ts lesion to construct stable double lesion mutants) is planned. Since previous studies have demonstrated that a mutant with a 39°C shutoff temperature or higher is too reactogenic in man, mutants with 40°C shutoff temperature and double lesioned 39°C

mutants will not be characterized further. Those ts mutants with a 37°C or 38°C shutoff as well as single lesioned 39°C mutants will be examined for genetic stability in vitro and subsequently in vivo. Those mutants that fail to lose their ts phenotype after replication in vivo and in vitro will be characterized further for the presence of a deletion in an RNA segment.

Studies of Influenza Virion RNA by Polyacrylamide Gel Electrophoresis - The influenza A virus genome is made up of 8 pieces of RNA that can be resolved by polyacrylamide gel electrophoresis (PAGE). Since the corresponding RNA segments from different strains may be induced to migrate at different rates by altering the conditions of electrophoresis, it is often possible to determine the parental origin of genes present in recombinant viruses. Determination of parental origin of genes in recombinant viruses permits the following types of studies: (1) assignment of ts lesions to specific RNA segments, (2) correlation of genetic behavior of ts viruses with location of ts lesion(s), and (3) determination of effect of gene constellations on biological properties such as level of attenuation, etc. This work is being conducted in collaboration with Dr. Francoise Thierry who is visiting the N.I.H. from the Pasteur Institute.

The single stranded RNA of influenza A virions is electrophoresed on a low concentration polyacrylamide gel (PAGE) in the presence of 4M to 6M urea. Temperature, concentration of urea, acrylamide concentration, and duration of electrophoresis all affect the ability to distinguish differences among RNA segments from different strains. Bands are visualized, after staining with ethidium bromide, by U.V. light activation.

Our approach to the use of ts mutants as vaccines is based on the assumption that genes bearing ts mutations are responsible for attenuation. Thus, genes contributed to ts recombinants by the wild type parent should not affect virulence appreciably. Attenuation of the HK ts-1[E] recombinant in animals and man correlated with ts mutations on P3 and RNP genes inherited from its ts Great Lakes/65 parent. Evaluation of Udorn/72 and Georgia/74 ts-1[E] recombinants suggested that the level of attenuation of ts-1[E] viruses was not influenced by those genes inherited from the virulent wild type parent virus that could not be scored by genetic or immunologic assay (i.e., P1, P2, M, and NS). Two Vic/75-ts-1[E] clones, 81 and 113, both bearing the $H3_{75}$ hemagglutinin and the $N2_{75}$ neuraminidase, retained some residual virulence for adult volunteers (about 10% febrile and/or systemic illness). Previously, ts-1[E] recombinants had been shown to retain some residual virulence in children who were doubly seronegative. Since these previous ts-1[E] recombinants were satisfactorily attenuated for adults with residual neuraminidase immunity, we hypothesized that absence of appreciable neuraminidase immunity was responsible for the reactogenicity of the A/Vic/75 ts-1[E] clones 81 and 113 in adult volunteers. Subsequent evaluation of clone 67, a Vic/-ts-1[E] strain that bore the 1965 neuraminidase, supported this view, since there were few reactions observed in those vaccinees who possessed significant levels of anti-$N2_{65}$ antibody. Nonetheless it was possible

that parental ts or wild type genes other than the ts-P3 or ts-RNP gene influenced the reactogenicity of clones 67, 81 and 113.

From a comparison of the genotypes of the three Vic/75-ts-1[E] clones, their virulent wild type parent (Vic/75) and Georgia/74ts-1[E] virus and the response of volunteers to these viruses the following conclusions were drawn:

(1) Viruses possessing the P3 and RNP genes derived from HK/68-ts-1[E] are at least moderately attenuated for man. Clone 81 bears all wild type genes except for the ts-1[E] P3 and RNP genes and is moderately attenuated.
(2) The possession of the wild type hemagglutinin (H) gene does not appear to affect virulence for seronegative volunteers.
(3) It is not the parental origin of the NA gene that affects the expression of reactogenicity but rather whether volunteers have antibody to the neuraminidase present on the vaccine virus. This follows from the observation that the Georgia/74-ts-1[E] virus bore wild type $N2_{74}$ but was attenuated in volunteers who had pre-existing NA antibody.
(4) The increase in reactogenicity noted for clones 81 and 113 compared to clones 67 and Georgia/74-ts-1[E] cannot be ascribed to the Vic/75 P1 and P2 genes, since clone 113 derived its P1 and P2 genes from ts-1[E] while clone 81 received both of these genes from the wild type parent.
(5) It is unlikely that the wild type Vic/75 NS gene contributed to the virulence of clones 81 and 113, since the Georgia/74-ts-1[E] virus also bears a wild type NS gene and was as satisfactorily attenuated as clone 67, which bears the NS gene of HK/68-ts-1[E] (also a wild type HK/68 gene).
(6) The role of the Vic/75 M protein gene in the retention of residual virulence by clones 81 and 113 could not be determined. Notwithstanding the possible effect of the Vic/75 M gene on the reactogenicity of clones 81 and 113, their residual virulence can best be explained by the very low level of neuraminidase antibody possessed by the vaccinees.

Current interpretation of the nature of the genetic defects in our single lesion ts mutant probes, both HK and Udorn, is based on extrapolation from the PAGE studies of Palese and Ritchey and others with WSN ts mutants of Sugiura and with our HK/68-ts-1[E] recombinant. We are seeking to expand this information. The RNA segments of the Udorn/72 and the WSN/33 wild type viruses have different electrophoretic patterns. When recombinants are made between WSN/33 and Udorn/72 viruses, it is relatively easy to determine by PAGE the parental source of each gene. Therefore, we mated our Udorn/72 and HK/68 single lesion ts mutants with WSN wild type virus under conditions which favored the emergence of H3 ts^+ recombinants. We hoped that the locus of a ts lesion could be defined by demonstrating that all H3 ts^+ recombinants shared a single gene from WSN wild type. This gene would necessarily represent the locus of the ts lesion in the original Udorn/72 or HK/68 single lesion mutant. Unfortunately, this approach yielded recombinants that bore predominantly WSN genes. Currently this problem is being approached in

an alternate manner, by selecting for WSN ts recombinants. By this method it should be possible to determine whether mutants that fail to complement share a ts lesion in the same gene, whether intracistronic complementation may occur between mutants that share a ts lesion, and whether our combined sets of HK and Udorn mutants are representative of the complete influenza A virus genome.

Production and Characterization of ts-1A2 Recombinant Viruses -When immunity to both surface antigens is lacking, only the degree of defectiveness of the vaccine virus determines attenuation and in this situation defectiveness must be greater than that specified by the ts-1[E] lesions. For this reason we constructed a recombinant (1A2) with a set of ts lesions that specified a greater degree of defectiveness than that seen with the ts-1[E] recombinants. Initially ts mutants of wild type influenza A virus were produced by chemical mutagenesis. Next, viruses with a single ts lesion were identified among the ts progeny of mutagenized virus or were produced by segregating recombinants with single lesions from mutants bearing two or more ts lesions. The single ts mutants were characterized as to complementation-recombination group and were evaluated for degree of growth restriction and genetic stability in vivo (in the hamster). In this manner we identified the 2 most defective and stable ts lesions that were genetically distinct, i.e., located on different RNA segments of the genome. Finally, we combined the 2 lesions into a single virus by genetic recombination. The resulting recombinant virus (designated 1A2) that had the A/Udorn/72 hemagglutinin and the desired two ts lesions (complementation groups 1 and 5) was more restricted in replication at 37°C than either ts parent or the ts-1[E] recombinants. Genetic evidence suggested that the 1A2 ts lesions were located on the gene coding for P3 protein and the gene coding for P1 protein; both of the affected genes are believed to code for large proteins thought to be involved in the synthesis of complementary RNA. Thus, the 1A2 recombinant has defects in both proteins required for the initial step in viral replication.

The double ts-1A2 recombinant exhibited an impairment in complementation and recombination not found in two parallel passaged single ts viruses which possessed either the group 1 or group 5 ts gene present in ts-1A2 virus. Interestingly, the HK/68-ts-1[A] virus and its Udorn/72-ts-1[A] recombinants that possessed both group 1 and group 5 ts lesions did not manifest the impairment in complementation and recombinant seen with ts-1A2. This suggests that the group 5 ts gene in the ts-1A2 virus differed from that in the HK/68 and Udorn/72 ts-1[A] viruses.

The reason for the impairment in complementation-recombination of the Udorn/72 ts-1A2 virus is not understood and can only be speculated upon. Possibly, many virions produced during a mixed infection involving the ts-1A2 virus are phenotypically mixed particles possessing both the ts P1 and P3 polymerase proteins. Such viruses, irrespective of their genotype, would have greatly decreased infectivity at restrictive temperature because c-RNA synthesis from the virion RNA would be restricted by the ts P1 and P3 proteins. The presence of approximately 15 P1 and P3 proteins per virion suggests that each gene segment has one or more P1

and P3 proteins. Thus, such phenotypically mixed particles could be restricted at high temperature if any one of the 8 viral genes had both the ts P1 and P3 proteins attached to it. In this manner, the lack of recombination seen between the ts-1A2 virus and other ts viruses may reflect the presence of the ts-1A2 defective proteins in recombinant viruses whose genotype is $\frac{ts}{+}$. Others have observed interference between a ts-virus defective in c-RNA synthesis and wild type virus although the mechanism of this interference was not determined.

Previously, it had been demonstrated that the extent to which ts recombinants were restricted in growth in the lower respiratory tract was a function of their temperature sensitivity as measured by shutoff temperature of plaque formation in tissue culture. The lower the shutoff temperature of a ts virus the lower the level of virus replication in the hamster's lungs. The A/Udorn/72 ts-1A2 virus, which had a 37°C shutoff temperature, failed to replicate in the lungs of hamsters and was significantly restricted in the nasal turbinates. Udorn/72-ts-1[E] recombinants, which had a 38°C shutoff temperature and ts lesions on genes represented by complementation-recombination groups 1 and 2, replicated in the hamster's lungs to a level intermediate between that of the ts-1A2 recombinant and wild type virus and were not restricted in the nasal turbinates. This indicated that the Udorn/72-ts-1A2 virus (37°C shutoff) was more restricted in the upper and lower respiratory tract than its ts-1[E] counterparts (38°C shutoff). Although Udorn/72-ts-1A2 was restricted in replication in both the upper and lower portions of the respiratory tract, infection with this virus provided significant protection against wild type virus challenge.

Genetic instability of a ts virus is most easily detected in sites of replication which are restrictive for growth, such as the lungs which have a temperature partially restrictive for most influenza A virus ts mutants. The replication of the ts-1A2 reombinant was sufficiently restricted at 37°C that virus did not grow in the lungs. However, all 22 isolates obtained from the nasal turbinates, an organ in which the ts-1A2 recombinant virus was partially restricted in replication, were ts. This suggested that the ts-1A2 recombinant virus was stable gentically in vivo.

The Udorn/72-ts-1[A] clone 189, which was the parental donor of the complementation group 1 ts lesion to ts 1A2, did not "revert" in vivo and thus was more stable than the HK/68-ts-1[E] virus (complementation groups 1 and 2 ts lesions) and its complementation group 1 segregant Udorn/72-ts-1[E] clone 13. This was so even though the group 1 ts gene present in Udorn/72 clone 189, HK/68-ts-1[E] virus, and Udorn/72-ts-1[E] clone 13 originally came from the same A/Great Lakes/65-ts-1 parent. These observations indicate that the genetic stability of this group 1 ts lesion can undergo change during recombination and/or passage. Further information concerning the in vivo genetic stability of the group 1 gene in the Udorn/72 ts-1A2 recombinant virus should emerge from an examination of a series of single lesion group 1 segregants derived

from it. Such an analysis is now underway.

Ideally a master strain which serves as the donor of attenuating genes should possess ts genes which are completely stable genetically in vivo. The group 1 ts gene of the ts-1A2 recombinant appeared to meet this requirement, but the group 5 ts gene exhibited some instability. However, together these two ts genes rendered the Udorn ts-1A2 recombinant genetically stable in vivo and thus the ts-1A2 genes may prove to be a satisfactory tandem set of ts lesions for use in a master strain. Genetic drift in the group 1 ts gene in other recombinants, described above, suggests that the genetic instability of this gene in the 1A2 complex may present a problem when 1A2 recombinants are prepared in the future. However, experience gained from preparation of Victoria/75, Alaska/77 and Hong Kong/77 ts 1A2 37°C shutoff recombinants indicates that other genetically stable recombinants can be produced using the Udorn/72-ts-1A2 as a donor virus. Thus, genetic drift of the group 1 ts gene is not inevitable, indicating it is possible to conserve the desired genetic stability of this gene during transfer and passage. Additional analysis of future recombinant viruses derived from the Udorn/72-ts-1A2 virus must be made to determine if there is a regular association between a 37°C shutoff temperature of recombinants bearing both ts-1A2 lesions and genetic stability in vivo. If this is the case, ts-1A2 recombinant vaccine viruses bearing the new surface antigens can be selected quickly and their genetic stability validated rapidly by tests in hamsters prior to initiation of clinical trials in man.

During the past year efforts were directed toward mating the A/Udorn/72-ts-1A2 double recombinant with the A/Vic/3/75 wild type virus and analyzing the progeny for shutoff temperature of plaque formation, ts genotype and level of replication in the respiratory tract of the hamster. The Udorn/72-ts-1A2 virus was mated with the influenza A/Victoria/3/75 wild type virus and 6 ts recombinant viruses bearing the Vic/75 hemagglutinin were isolated. Five of these Vic/75-ts-1A2 recombinants had a 37°C shutoff temperature and two ts lesions like their Udorn/72-ts-1A2 parent. Each of these clones replicated to low titer, or not at all, in the hamster's lungs and were 100-fold restricted in the nasal turbinates like their ts parental virus. This degree of growth restriction was greater than that seen with A/Vic/75 ts-1[E] recombinants or any of its predecessors. Each of 158 isolates recovered from hamsters infected with the parental Udorn/72-ts-1A2 virus or one of its Vic/75 ts double lesion recombinants retained the ts phenotype. One Vic/75-ts-1A2 recombinant clone that had a 38°C shutoff temperature and only the group 1 ts lesion replicated in the hamster's lungs to a level intermediate between that of the parental ts-1A2 and wild type virus. All isolates recovered from hamsters infected with this recombinant were also ts. Infection of hamsters with a Vic/75-ts-1A2 recombinant, bearing the group 1 and group 5 ts mutations, induced resistance to homologous wild type virus challenge. Since the parental and double lesion recombinant ts-1A2 viruses had similar restriction of replication in vitro at 37°C and in vivo, it is likely that the two ts-1A2 ts lesions will effect a similar level of

attenuation following transfer into other antigenic variants of influenza A virus. These data provided the basis for evaluation of these promising recombinants in volunteers.

The ability of the ts genes of the Udorn/72-ts-1A2 virus to reproducibly attenuate additional influenza A wild type viruses within the H3N2 subtype was evaluated further by mating this donor with the A/Alaska/77 (H3N2) wild type virus. Fifteen recombinant viruses possessing the Alaska hemagglutinin and the ts phenotype were characterized. Each of 4 Alaska/77-ts-1A2 recombinant viruses that possessed both ts-1A2 ts genes had a 37°C shutoff temperature. Nine viruses that received only the complementation group 1 gene (P3) exhibited a spectrum of shutoff temperatures ranging from 37° to 39°C. Two clones had the isolated group 5 ts gene (P1). One had a 38°C shutoff temperature and the other a 39°C shutoff temperature. The level of replication of 9 of these recombinant viruses was evaluated in hamsters. Each of the 4 ts-1A2 recombinants with a 37°C shutoff temperature and both ts genes grew to a level comparable to their Udorn/72-ts-1A2 parent virus in the lungs and nasal turbinates, i.e., they were approximately 10,000-fold reduced in growth in the lungs and 100-fold in the nasal turbinates. Three group 1 viruses were also evaluated: two 37°C shutoff mutants resembled the 37°C double ts Alaska/77-ts-1A2 recombinants in restriction of growth in vivo; the 38°C shutoff mutant grew in the lungs to a level intermediate between that of the ts-1A2 (37°C) and wild type (39°C) viruses. Similarly, the group 5 mutant with the 38°C shutoff temperature was more restricted in pulmonary replication than the 39°C group 5 mutant. Identification of both group 1 and group 5 segregants among the A/Alaska/77 recombinant viruses confirms that the Udorn/72-ts-1A2 indeed has two ts lesions. Furthermore, our experience with the A/Alaska/77 recombinants provides additional evidence that ts-1A2 recombinant viruses that possess both ts-1A2 lesions have a predictable set of genetic and biological properties.

The Udorn/72-ts-1A2 virus was also mated with the A/HK/123/77 (H1N1-Russian flu) virus and three recombinant ts-1A2 viruses that had both a 37°C shutoff temperature and the group 1 and 5 ts genes were obtained. These recombinants resembled their A/Udorn/72 ts-1A2 parent in restriction of replication in the nose and lungs of the hamster. These preliminary results suggest that viruses within the H1N1 subtype can also be predictably attenuated by the acquisition of the two ts-1A2 lesions.

Comparison of the Genetic Properties of the 5FU Induced Mutants and Cold Adapted Variants of Influenza - An alternate approach to the use of mutagen induced ts mutants as donors of genetic lesions to attenuate influenza virus vaccine strains is to use cold adapted viruses as the donors of attenuating genes. Maassab, Kendal, and Davenport and their collaborators have developed a series of cold mutants and cold recombinants that can replicate equally well at 25° and 33°C in tissue culture. Wild type influenza viruses do not replicate efficiently at 25°C, but can be adapted to grow at low temperatures either by a stepwise process in which they are passaged at successively lower temperatures (differing by

3°C) or by blind passage at 25°C for several cycles followed by plaque selection at 25°C. Transfer of the cold adaptation (ca) lesion(s) is carried out at 25°C so that with the addition of appropriate antiserum, recombinants with the ca property can be selected directly. Using this methodology Maassab has made extensive use of the cold adapted parent PI-7 (derived from the A/AA/6/60-H2N2 strain) as a donor of the ca property to a number of other antigenic variants of influenza A virus.

Initial studies of the parent strain (PI-7) and 12 of its cold recombinants--one clone of CR 6 (Queensland/6/72-H3N2), one clone of CR 12 (A/AA/9/73-H3N2) and 10 clones of CR18 (A/Scot/840/74 H3N2)--showed that the parent and the 12 recombinants were temperature sensitive for plaque formation in addition to being cold adapted. They were also shown by complementation-recombination to share our complementation-recombination group 1 ts lesion. The 12 recombinants showed a spectrum of shutoff temperatures for plaque formation with CR6 and 4 of the CR 18 clones sharing the 37°C shutoff temperature of the PI-7 parent, and the CR 12 virus and 6 of the CR 18 clones having a shutoff temperature of 38°C. In addition, one clone of a fourth recombinant CR 13 (A/Dunedin/4/72-H3N2), was not temperature sensitive.

If the PI-7 parent were to be used routinely as a donor of mutant genes for attenuation of new antigenic variants of influenza A virus it was necessary to study the products of multiple genetic transfers in order to verify that the desired biological and genetic properties were consistently transferred. To this end we examined the progeny from 5 additional matings of the PI-7 parent; CR 22 (Vic/3/75) - 18 clones, CR 24 (A/NJ/8/75)-16 clones, CR28 (Alaska/5/77)-2 clones, CR29 (Alaska/5/77 cloned)-5 clones, and 2 additional clones of CR 13. All of these ca recombinants were temperature sensitive. The shutoff temperatures varied: the 2 CR 13 clones had a shutoff temperature of 39°C; several clones of CR 22 and CR 24 appeared to have a 37°C shutoff temperature, while other clones of both of these recombinants had a 38°C shutoff temperature. Although all of the recombinants of CR 22, CR 24, CR 28 and CR 29, were temperature sensitive some of them underwent genetic interaction with our Hong Kong (HK) group 1 ts mutant prototype in addition to complementing ts mutants representing the other 6 complementation groups. This suggested these recombinants had a ts lesion not represented in our 7 complementation groups. This ts lesion should also be present in the parental PI-7 virus. An attempt has been made to demonstrate the presence of 2 ts lesions, (i.e., the group 1 ts lesion and the other ts lesion) by carrying out complementation studies with the PI-7 virus and its recombinants and using as additional genetic probes a series of ts mutants of the WSN strain.

Genetic Analysis of the ts Lesions in Cold Variants and Their Cold Recombinants

Sugiura, Kilbourne, Ueda, Tobita, Krug and their collaborators have developed and characterized a series of 5FU ts mutants of the WSN (HON1) strain of influenza A virus. These mutants were assigned to 8 groups by complementation-recombination analysis, and the biochemical defect in

members of each group was established. Palese and Ritchey and their collaborators have recently been able to assign each genetic defect to a particular RNA segment by polyacrylamide gel analysis. When we compared the HK mutants with the WSN mutants using the complementation recombination assay, we were able to show that HK 1=WSN 1 (cRNA defect); HK 6=WSN 2 (vRNA defect); HK 5=WSN 3 (cRNA defect). Later Palese demonstrated, by using polyacrylamide gel electrophoresis that the HK 2=WSN V (vRNA, RNP defect). The other 3 HK and 4 WSN groups did not appear to share lesions based on their ability to undergo genetic interaction in the complementation recombination assay. In this assay, pairs of ts mutants are tested for their capacity to produce plaques with high efficiency during mixed infection at restrictive temperature. If neither virus by itself is able to produce a significant number of plaques at restrictive temperature a positive result during mixed infection is interpreted as evidence of genetic interaction. Since complementation might occur in the absence of recombination (for reasons discussed below) we have attempted to define the basis for the observed interactions by: (i) analyzing the progeny of plaques that appeared at 39°C (the nonpermissive temperature) on dually infected monolayers for their efficiency of plating at 33° (a permissive temperature) and at 39°C; (ii) carrying out recombination with various virus pairs by mixedly infecting cells at 33° and then assaying the virus yield for the presence of virus that can plaque at 39°C (an indication of the occurrence of recombination). Results of such tests should help to determine whether complementation in the absence of recombination was occurring. To date this work has been hampered by the fact that the WSN mutants have two characteristics that make genetic analysis difficult: (i) they have a tendency to be leaky, i.e., plaques form in singly infected control cultures incubated at the non-permissive temperature and (ii) at least one of them has a tendency to interfere during tests for genetic interactions. [Thus, despite the fact that one or both of the viruses is leaky (i.e., able to grow to low titer in singly infected cultures incubated at the non-permissive temperature), there may be no growth of virus in dually infected cultures, despite the fact that other methods have shown a particular pair of mutants not to share ts lesions.] Despite these difficulties we have obtained preliminary evidence from analysis of progeny from plaques produced at restrictive temperature during mixed infection suggesting that the HK4=WSN IV (neuraminidase defect) and that the HK3=WSN VIII (nonstructural protein defect).

Previous studies with cold variants and our HK set of ts mutants indicated that 5 independently derived cold variants that are temperature sensitive may share one or several lesions with the HK set: A/AA/6/60, group 1; A/AA/2/65, group 1; A/AA/2/67, groups 1,3,5; A/Aichi/2/68; groups 1, 6 and A/AA/1/70, groups 1, 3, 6. The purpose of comparing the genetic lesions of the cold variants and the WSN mutants was 2-fold: (i) to confirm the lesions identified by complementation with the HK mutants, in those cases where there was sharing of lesions between the HK and WSN groups and (ii) to ascertain whether the cold variants had any lesions in addition to those determined in tests with the HK prototype mutants, since there were several WSN and HK groups whose lesions that did not

overlap by complementation-recombination assay.

Again these studies were hampered by the 2 properties noted above for the WSN strains, i.e., the WSN mutants tended to interfere and to leak. The situation turned out to be more complex than was first anticipated. It was anticipated that cold variants that shared a lesion with a HK mutant would not interact by complementation with a WSN mutant that corresponded to the HK mutant. However, this was not always observed. Viruses that shared the HK group 1 lesion but which interacted with WSN group I could be divided into two subets: (i) one subset, including A/AA/6/60 and A/A/2/65, interacted with both members of the WSN I group (ts-1 and ts-6); (ii) the second group including, A/AA/2/67, A/Aichi/2/68 and A/AA/1/70 showed genetic interaction with ts-1, but not with ts-6. In the case of the ca virus that shared the HK group 5 lesion, (A/AA/2/67) there did not appear to be genetic interaction with WSN group III that also shared this lesion. Alternatively the A/AA/6/60 virus interacted with HK5 and not with WSN III. In the case of the viruses that shared the HK group 6 lesion (A/Aichi/2/68 and A/AA/1/70) there was evidence for genetic interaction between both of these viruses and the corresponding WSN group II representative. Three of the cold variants shared the WSN-V lesion and all of them underwent some genetic interaction with the corresponding HK group 2 mutant. However, the interaction of one of them (A/Aichi/2/68) was variable and that of the other 2 (A/AA/2/67 and A/AA/1/70) was of a low level. Several of the cold variants did not undergo genetic interaction with ts-11, a member of the WSN group IV which was subsequently shown to have a defect in the nonstructural protein in addition to a lesion in the neuraminidase gene. However, they all underwent genetic interaction with ts-3 a second member of group IV which appeared to have only a single lesion in the neuraminidase gene.

Examination of the A/AA/6/60 recombinants indicated that none underwent genetic interaction with the WSN group III. However, these ca viruses interacted with the HK group 5 ts prototype which appears to be equivalent to WSN III by both complementation-recombination and RNA polyacrylamide gel analysis.

Interpretation of Findings

The basic premise of complementation-recombination studies is that in dually infected cells, viruses which share ts lesions are not able to produce a functional protein corresponding to a particular RNA segment, whereas viruses which do not share ts lesions will each produce a functional protein corresponding to one that is defective in the other mutant with the resultant production of wild type progeny. However, misleading results can be obtained if one of the following phenomena occur: (i) intracistronic complementation, (ii) leak of one or both of the mutants, (iii) interference by a defective polypeptide in multimeric structures such as hemagglutin, neuraminidase or RNP complex, (iv) interference brought about by polypeptide-polypeptide incompatability in

multi-component structures in which proteins must interact. The first 2 of these phenomena lead to "too many" complementation groups, whereas the second 2 result in detection of "too few" groups. It should be noted that almost all of the gene products of influenza are either multimers themselves: the hemagglutinin, the neuraminidase, the matrix or are involved in a multicomponent RNA synthesizing complex: P1, P2, P3 and the nucleocapsid protein. Thus, the phenomera described above might be anticipated.

In the studies described above we have seen some evidence for each of these:

(1) Intracistronic complementation may explain the interaction of the cold variants with the HK group 1 and WSN group I mutants. All 3 of these types of mutants may share a lesion on the gene encoding P3, the viral transcriptase. The interaction of R1 (HK group 1) and ts-6 (WSN group 1) probably produces a non-functional protein as does that of ts-6 and A/AA/2/67. On the other hand, the interaction of the WSN ts-1 and the A/AA/2/67 ts polypeptides may produce a functional heteropolymer. The same mechanism could also explain the observations with WSN-II and HK-6 and WSN-III and HK-5.

(2) Attempts to examine the progeny virus from complementation plaques was hindered by the fact that the WSN mutants were leaky. Although one might anticipate finding ts virus only in plaques from a cross of ts-1 and A/AA/2/67 this was not the case because of the emergence of ts revertants.

(3) Interference appears to be a problem when one attempts to demonstrate complementation between WSN-V and HK-6. These viruses each appear to have lesions on different segments. Nonetheless, in the same test in which plaques developed on monolayers infected with WSN-V virus, plaques failed to develop on monolayers inoculated with both viruses. Interestingly both of the viruses have lesions in genes affecting v-RNA synthesis.

(4) Protein-protein incompatability also appears to be a deterant to genetic interaction. When the complementation ratios of WSN mutants tested against each other or against HK mutants were compared it was seen that the ratios in homologous tests were significantly higher suggesting inefficiency of polypeptide-polypeptide interaction between strains which were isolated over 30 years apart.

Relationship of the cold adaptation property to ts lesions - Since the complementation recombination studies did not enable us to establish a correlation of a specific ts lesion with the ca property, we sought to study this relationship by attempting to transfer the cold adaptation and temperature sensitive properties from a particular cold variant to a wild type strain of influenza A with different surface antigens. The properties of the viruses derived in this manner were defined in terms of their cold adaptation, their temperature sensitivity and the nature

of the ts lesion (or lesions) transferred. Recombinants were prepared at 25°C or at 33°C. They have not been completely characterized, but the following observations were made. The cold adapation property and the temperature sensitive property can be dissociated from each other. This dissociation occurs most readily when recombination is carried out at 33°C. It is also possible to derive recombinants that retain temperature sensitivity, but do not have the HK group 1 lesion. In addition, production of recombinants between a ts virus bearing the group 1 lesion and a cold variant that was not temperature sensitive (A/FM/1/47-H1N1) yielded recombinants with varying degrees of cold adaptation; these recombinants also behaved as if they had ts lesions in HK complementation group 1, 3, 5 or 6. These findings suggest that during the process of recombination at 25°C two phenomena may occur: (i) selection during reassortment of genes coding for cold adapted polypeptides derived from the cold adapted parent and (ii) selection of spontaneous ts mutations affecting genes derived from the wild type parent. The genes most often involved are those coding for proteins in the RNP complex, specifically those involved in c-RNA synthesis. Such selection might be anticipated since without c-RNA synthesis the replicative cycle of influenza A cannot begin.

Animal studies with cold adapted variants and recombinants - The A/AA/6/60 cold adapted parent and four of its recombinant clones CR6, CR12, CR18 clones 6 and CR18 clone 7 were evaluated for replication in the lungs and nasal turbinates of hamsters. Each virus appeared to be attenuated; genetic stability correlated with the level of viral replication in the hamster lung, i.e., viruses which grew best showed a tendency to revert to the ts^+ phenotype. Characterization of the ts^+ revertants for the presence of the cold adaptation property revealed that these viruses exhibited a spectrum of cold adaptation properties. Two viruses, PI-7 (the parental cold variant) and the CR6 recombinant (A/Queensland/6/72) did not revert in either the lungs or nasal turbinates of hamsters.

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PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
STUDIES ON MYCOPLASMA PNEUMONIAE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. Michael B. Grizzard, LID, NIAID
Robert M. Chanock, LID, NIAID, Chief

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Infectious Diseases

SECTION
Respiratory Viruses Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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SUMMARY OF WORK (200 words or less - underline keywords)

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PERIOD COVERED
October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Laboratory and Epidemiologic Studies of Viral Hepatitis Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

This project consists of continuing studies of the chemistry, structure, epidemiology, immunology and pathology of the human hepatitis viruses. The goal of such studies is the control of human viral hepatitis by application of the most appropriate methods, including active and passive immunization, chemotherapy and interdiction of spread of the viruses. Progress: The biophysical and biochemical characterization of hepatitis A viral antigen has begun, and studies of the immunopathology of hepatitis type A in non-human primates, using defined pools of virus, are in progress. An inactivated subunit vaccine for hepatitis type B has been developed and is undergoing extensive tests of safety and efficacy in chimpanzees and man. A third hepatitis B antigen, e antigen, is being characterized and its relationship to infectivity is being explored. Evidence that populations of hepatitis B viruses may contain defective interfering particles has been obtained, and this finding is being utilized in renewed attempts to isolate the virus. A newly recognized clinical syndrome, type non-A, non-B hepatitis has been further defined and attempts to identify an etiologic agent intensified through transmission studies in chimpanzees.

Project Description

Objectives: (1) To collect clinical material of high potential infectivity for use in laboratory studies, (2) To develop and test in vitro procedures and animal systems for use in laboratory studies of viral hepatitis, (3) To propagate the viruses of hepatitis in culture or in experimental animals, (4) To study the biochemistry, biophysics, immunology and epidemiology of hepatitis-associated antigens; (5) To develop vaccines of other control measures for viral hepatitis; (6) To develop chemotherapeutic approaches to the control of chronic viral hepatitis.

Methods Employed

(1) Longitudinal collection of clinical specimens from hepatitis patients.

(2) Ultracentrifugal, electrophoretic and column chromatographic purification of hepatitis viruses and related antigens.

(3) Inoculation of non-human primates for the determination of infectivity and clinical spectrum of hepatitis viruses.

(4) Microscopic examination of hepatitis viruses and virus-infected tissue by fluorescence microscopy and electron microscopy.

(5) A wide variety of tissue culture techniques.

(6) A wide variety of immunologic techniques for measuring humoral and cellular immune response.

Major Findings

(1) Type B Hepatitis

HBs Ag

Because of its potential usefulness as the raw material for hepatitis B vaccine, HB_sAg is being intensively studied. Preparation of two pilot lots of vaccine, one subtype adw and the other subtype ayw, has been completed by Dr. J. Gerin, on a collaborative program. Following extensive safety testing in chimpanzees, both vaccines are currently undergoing safety testing in humans. Safety and efficacy tests of a bivalent vaccine prepared from the two monovalent vaccines has been completed, and, upon completion of the safety tests of the monovalent vaccines in humans, will be subjected to immunological evaluation in volunteers in late 1978. If all goes well, it is anticipated that efficacy studies will begin in March, 1979. Baseline epidemiology for these efficacy trials is currently being studied.

Although a hepatitis B vaccine prepared from HB_sAg purified from the plasma of chronic carriers of the antigen is feasible and probably cost-effective for its contemplated uses in the United States, such a vaccine probably will not be cost-effective for the people most in need of a vaccine in the

developing world. Therefore, additional studies on alternative methods of purification and inactivation, comparisons of monovalent vs. bivalent or polyvalent vaccines, studies of different combining ratios of mononvalent vaccines into bivalent preparations, different vaccine dosages, different vaccination schedules, different routes of administration, studies of possible adjuvants and their interaction with vaccine preparations and further characterization of the immunizing antigens themselves will be carried out. In addition, one important observation associated with the vaccine trials in chimpanzees was that type B hepatitis could be prevented in chimps by vaccination after exposure. Although based upon limited data, there was evidence that such post-exposure vaccination might be more effective than administration of hepatitis B immune globulin following exposure. Additional studies in chimpanzees to confirm and extend these observations are planned.

Other HBV Antigens

HB_cAg - Current interest in HB_cAg centers on the association between its detection in liver cell nuclei and the prognosis and relative infectivity of chronic hepatitis type B patients. A commercial test for anti-HB_c, based upon a radioimmunoassay first described by us, will soon be available.

HB_eAg - There has been much interest in this non-particulate hepatitis antigen because of its association with chronic hepatitis and relative infectivity of blood from HB_eAg positive type B hepatitis patients. Firm proof that HB_eAg is a hepatitis virus gene product is lacking, but circumstantial evidence strongly points in that direction. There have been reports that HB_eAg is a) on the surface of hepatitis B virions, b) an idiotypic IgG molecule and c) a modified lactic acid dehydrogenase. Probably none of these is correct: it is probably either a nonstructural viral gene product or excess viral DNA polymerase that is never assembled into complete virus. We have recently developed procedures for the purification of HB_eAg, and preparations in which host serum proteins cannot be detected have been prepared. Plasmapheresis units obtained from chimpanzees chronically infected with HBV have served as raw material for the purification. We plan soon to scale up the purification procedures, which require solid immunoabsorbent columns and ultracentrifugal separations, to prepare large enough quantities for characterization and hyperimmunization of animals. Such hyperimmune serum will be used in attempts to develop specific radioimmunoassays and immunofluorescence assays, as well as reference reagents.

"Delta antigen" - This antigen was discovered approximately a year ago by Rizzetto in Italy. It appears to be another hepatitis B antigen found, like HB_cAg, in the nucleus of infected liver cells. It has been detected only by immunofluorescence to date, but its existence has been confirmed by others. Because of the importance of thoroughly evaluating vaccine recipients for all possible markers of hepatitis B virus infection, we plan to characterize this new antigen and determine its relationship to type B hepatitis. Dr. Rizzetto is spending a year in Dr. Gerin's laboratory and my laboratory for

this purpose. Activities will include studying the development of delta antigen and anti-delta antigen in experimentally infected chimpanzees, determining its relationship to other hepatitis B antigens, attempting to purify the antigen from infected liver tissue (human and chimpanzee) and the preparation of reagents. This work is progressing rapidly: a radioimmunoassay for delta antigen and antibody has been developed and is being applied to seroepidemiological studies.

Hepatitis B Virus

Because HBV occurs in titers as high as 10^8 infectious particles/ml in the plasma of certain chronically infected individuals, it is possible to purify these particles by large volume ultracentrifugation and characterize them. Recent collaborative studies carried out with Dr. Gerin have revealed that HB virions are heterogeneous: three populations of particles can be separated by isopynic banding in cesium chloride. The lowest density population lacks DNA polymerase activity and probably most if not all nucleic acid. The highest density population is strongly positive for polymerase activity and contains double-stranded circular DNA that can be extracted and characterized. Most interesting is the intermediate density population which, itself, is heterogeneous and which is weakly polymerase positive and contains nucleic acid with a molecular weight only 80-90% of that of the highest density particles. Thus, these particles have all of the biophysical characteristics of defective interfering particles, entities that in other classical virus systems have been shown to inhibit fully infectious virus synthesis *in vitro* and to modulate virus infection *in vivo* in certain laboratory animal model systems, converting a rapidly progressive infection to a persistent or chronic one. Such a defective interfering particle system has never been described for a human disease. This finding has important implications for the two following topics.

Isolation of HBV *in vitro*

Despite many attempts over many years by many investigators, HBV has never been successfully isolated and serially transmitted *in vitro*. The reason for this is not known and may be related to a number of factors including limited host range (HBV is known to grow only in hepatocytes of humans and certain species of apes closely related to man). However, one reason for failure to isolate the virus may be that the virus is always found in the presence of a vast excess (up to a million-fold more particles) of viral coat material (HB_sAg) as well as a variable concentration of defective interfering-like particles. The former can compete for receptor sites on sensitive cells, and the latter can inhibit the synthesis of infectious virus when they coinfect cells with intact virions. To examine this, preparations of high density and intermediate density HB virions are being purified by isopynic and rate zonal centrifugal procedures under conditions of high containment in Dr. Gerin's laboratory. These preparations are being aliquoted and titered for infectivity in chimpanzees; they will be used for attempts to isolate HBV in a number of tissue culture cell lines.

Coincident with these studies is the development of reagents for identifying all of the markers of HBV infection. These assays will be used in evaluating various clones of hepatocytes derived from fetal human or chimpanzee liver. Cell lines that can be certified as being of liver origin will be inoculated with partially purified HBV that is being titered in chimpanzees. These inoculated cultures will be monitored with the various assays available in the laboratory including radioimmunoassays for HB_sAg and HB_cAg, HBV-specific DNA polymerase enzyme activity, visualization of HB_sAg and HB_cAg particles and HB virions, immunofluorescent detection of HB_sAg or HB_cAg and detection of intracellular HBV genome by nucleic acid homology techniques.

Although HBV has not been successfully cultured in vitro, recently a hepatoma cell line was isolated that synthesizes HB_sAg. This cell line, derived in South Africa, has recently been distributed to others, and we are beginning a detailed characterization. It is recognized that this cell line cannot serve as a source for hepatitis B vaccine because of its malignant origin, but it may yield important information on HBV-cell interaction. Attempts to recover the virus by cocultivation and induction with anti-metabolites are in progress.

Pathology

Although much has been learned about hepatitis B virus and its antigens, the method by which it produces disease is still poorly understood. There is some evidence that HBV is not cytopathogenic but, rather, produces tissue damage through the host's immune response to infection. Thus, it has been postulated that the host's humoral or (more probably) cellular immune response to viral antigens or modified liver cell antigens may lead to cell damage or death. Without going into detail here, there is evidence both for and against this hypothesis. However, attempts to prove the immunological nature of viral hepatitis have been unconvincing for a number of reasons, including difficulty in measuring and interpreting cellular immune responses in man. It is unlikely that this question can be adequately answered until autologous target cells infected with HBV can be maintained in vitro and lymphocytoxicity between these cells and lymphocytes obtained prior to infection, during the acute phase of illness and during convalescence can be studied in vitro. This is not technically feasible at present, but studies of the possible role of anti-idiotype antibodies in modulating HBV infection are feasible and will be attempted.

However, there is an alternative explanation for the variable course of type B hepatitis: the infection may be modulated by the ratio of defective interfering-like particles and fully infectious virus. This hypothesis is amenable to experimental analysis. We plan to experimentally infect chimpanzees and obtain serial plasmapheresis units from the animals throughout the course of infection. These plasmapheresis units will be analyzed for the ratio of the various density populations of HB virions as a function of phase of the disease and the results correlated with analyses of serum HB_sAg and HB_eAg titers, serum liver enzyme levels, intrahepatic HB_cAg and HB_sAg and histologic evidence of liver damage.

If the results of these experiments warrant it, consideration will be given to combining purified heavy density and intermediate density HB virions in different ratios and inoculating chimpanzees to determine if the clinical course of type B hepatitis in the animals can be predicted.

Meyer zum Buschenfelde produced a chronic hepatitis in rabbits by exhaustive immunization with liver tissue. He demonstrated that an antigen on the surface of hepatocytes (liver specific protein, LSP) was responsible for initiating this immunological disease. A similar LSP-anti-LSP system was shown to be present in cases of human chronic hepatitis. In independant studies and in studies in collaboration with our laboratory, Meyer zum Buschenfelde has shown that such anti-LSP-positive chronic hepatitis patients appear to have a disease distinct from hepatitis B virus-induced chronic hepatitis. However, this has not been rigorously proven, and the published concern that hepatitis B vaccine hypothetically contaminated with LSP might lead to chronic hepatitis in vaccine recipients has led us to begin studying LSP in more detail. LSP appears to be an extremely labile membrane-associated antigen that has not been purified, although one group in England believes that they have effected a purification. Earlier reports of various assays for LSP are now suspect, and the demonstration of LSP on liver cell surfaces by indirect immunofluorescence appears to be the only reliable assay method at present. We have recently established this assay in the laboratory and are beginning to study this antigen-antibody system. There is a great need for the development of more sensitive and less cumbersome tests for measuring LSP and anti-LSP and for the preparation of hyperimmune serum. If sensitive assays such as radioimmunoassays can be developed for LSP and anti-LSP, they will be applied to serial serum samples from chimpanzees and humans with type B hepatitis (and other types of hepatitis) to determine if there is evidence for this type of immunological response in viral hepatitis. The results will go a long way toward resolving the question of whether hepatitis is an immunological disease.

Antivirals and Chemotherapy of Type B Hepatitis

With the almost simultaneous reports of modification of chronic hepatitis B virus infection in humans or chimpanzees by three laboratories (ours, Merigan's at Stanford, and Desmyter's in Belgium) in 1976 a new era in type B hepatitis research began. Both interferon and an interferon inducer were shown to markedly diminish all of the markers of hepatitis B virus infection when they were administered in sufficient doses and for a sufficiently long period of time. Subsequent studies have shown that interferon is not the "magic bullet" for treatment of chronic hepatitis, and there is still insufficient information about the toxicity of certain interferon inducers to use them in large doses in humans, but the studies did show that the treatment of chronic hepatitis can be approached from the standpoint of treating the virus rather than the host. All previous treatment regimens have been concerned with suppressing the host's immune response with steroids and antimetabolites in an attempt to diminish the clinical manifestations of disease. These studies have recently been critically reviewed by the Washington, D.C.,

VA liver group and their conclusion was that steroids and antimetabolites have not been shown to be effective in the treatment of chronic type B hepatitis, a finding in agreement with the observations of others.

Further evidence that chemotherapy holds promise for the treatment of chronic type B hepatitis comes from recent studies by Merigan in which the antiviral drug adenine-arabinoside (Ara-A) was also shown to markedly diminish the titer of the various markers of hepatitis B virus infection in two patients with chronic type B hepatitis. However, there was significant toxicity associated with treatment with this drug.

An important aspect of the interferon/interferon-inducer studies is that a chronic virus infection was modified. In in vitro and in vivo studies of the action of interferon this substance has been found to be relatively ineffective after infection of cells has taken place: its primary mode of protection is by preventing spread of virus from infected cells to uninfected cells. This has very important implications for the understanding of chronic type B hepatitis and suggests that such chronic hepatitis B virus infection is an ongoing disease in which infected cells produce new virus, die and are replaced by new cells that are subsequently infected with virus and pass through the same cycle. Interruption of that cycle of synthesis of new virus and transmission to uninfected cells may be the key to terminating chronic type B hepatitis. This becomes even more significant in light of one of the key observations in our interferon-inducer study in chimpanzees: we were able to show a marked increase in the ratio of defective (polymerase-negative) hepatitis B virions to polymerase-positive virions as a result of treatment of the chimpanzees with interferon inducer and at a time that coincided with the fall in titer of the other markers of hepatitis B virus infection. This would be consistent with our hypothesis that chronic type B hepatitis is a defective-interfering virus system in which modulation of the infection can be achieved by altering the ratios of infectious and defective particles. Verification of this is technically possible by the methods described above and by treating chronically infected chimpanzees with interferon, interferon inducers or other antivirals and monitoring the ratios of different hepatitis B virions by sophisticated isopycnic banding procedures.

Other Studies

Although much has been learned about the epidemiology of type B hepatitis in recent years, there are several intriguing questions still unanswered. One stems from the discovery that approximately 0.1% of the United States population are carriers of HB_sAg; a proportion of these have associated chronic persistent hepatitis or chronic active hepatitis, the latter a life-threatening disease. What is totally unknown, however, is what proportion of the population has evidence of chronic hepatitis of any etiology. There is evidence that, as with hypertension, there is a large reservoir of undiagnosed disease not recognized because it has not been systematically sought. In collaboration with others, we are initiating a study of the Framingham population for evidence of occult chronic

hepatitis. When identified, such patients will be evaluated and the etiology of their hepatitis determined. If the results of initial surveys warrant it, we would attempt long-term followup of the Framingham population in an attempt to determine the incidence and outcome of such disease by etiology.

(2) Hepatitis A Virus

Characterization of the virus

Although hepatitis A virus (HAV) has not been isolated in vitro, sufficient particulate viral antigen(presumably HAV) has been available to a very few laboratories to begin biophysical and biochemical characterization. Two preliminary studies indicate that the viral particles have peptides similar to those of picornaviruses. Although definitive nucleic acid studies have not been completed, two studies suggest that the viral nucleic acid is RNA but possibly of lower molecular weight than that of picornaviruses. It is anticipated that, when and if HAV is cultured in vitro, the purification techniques recently developed will be directly applicable to vaccine preparation.

Antigen(s)

Only one antigen, hepatitis A antigen (HAAg) has been associated with type A hepatitis infections to date. Four serologic techniques for detecting this antigen and antibody to it have proven most useful for serologic studies of type A hepatitis infection: these are immune electron microscopy, immune adherence hemagglutination, radioimmunoassay and enzyme-linked immunoassay. Recently we have developed an immunofluorescence assay for hepatitis A antigen that has permitted us to study the tissue distribution of viral infection in chimpanzees and marmosets. HAAg was detected in the liver, germinal centers of lymph nodes and spleen, and basement membrane of the kidney. The latter sites probably represent sequestration of antigen released from the liver into the circulation. Interestingly, viral antigen has not yet been detected in the gut. However, only animals intravenously infected have been examined to date. We are presently studying gut tissue obtained sequentially from marmosets experimentally infected with HAV via the oral route.

Much of our current knowledge of the epidemiology of hepatitis A virus infection comes from collaborative studies carried out with many investigators but predominantly Dr. Wolf Szmuness of the New York Blood Center. It is anticipated that additional studies will be carried out in many laboratories as soon as a recently developed commercial radioimmunoassay for hepatitis A viral antibody is licensed. The radioimmunoassay is patterned after the radioimmunoassay for anti-HA developed in this laboratory.

Although it is assumed that the 27nm virus-like particles associated with HAAg are the hepatitis A virions, rigorous proof of such identity is lacking. Establishment of identity by purifying the particles and demonstrating

that infectivity is associated with them requires proof that infectivity is not also found elsewhere in gradients, etc. or that the titer of infectivity is significantly higher in fractions containing the particles than in other fractions. Such an approach is difficult if not impossible with only chimpanzees and marmosets as indicators of infectivity. Pools of HAV have only recently been prepared and are currently being tested in chimpanzees and marmosets for titer of infectivity.

Isolation of the virus

As with HBV, HAV has resisted isolation attempts. The reason for this is not understood, but HAV particles with different densities in cesium chloride have been described. Whether type A hepatitis represents a defective-interfering particle system or whether the different densities are the result of cleavage of capsid peptides as occurs with parvoviruses and picornaviruses remains to be determined. Large scale purification procedures recently developed by Dr. Moritsugu may help to answer these questions.

The recent development of an immunofluorescence technique for HAAg has provided another powerful tool for evaluating attempts to isolate HAV in vitro. That HAAg can be detected in cultured cells was very recently shown in a collaborative study with the Bureau of Biologics: marmoset hepatocytes obtained during acute HAV infection were cultured in vitro, and HA Ag was detected in the cytoplasm of the cells through their first two in vitro passages. The specifity of the antigen in these cells was confirmed. By third passage the hepatocytes no longer contained detectable HA Ag. Although these cells, like hepatocytes from patients with type B hepatitis, lost their viral antigen upon repeated subculture, they provide an opportunity to study other approaches to isolation, including cocultivation, induction with anti-metabolites, use of helper viruses, etc.

Pathology

As with type B hepatitis, pathologic mechanisms involved in type A hepatitis are not understood. It appears that type A hepatitis rarely if ever progresses to chronic disease. However, this assessment is based upon a limited serologic analysis of chronic hepatitis patients. It is now possible to directly examine liver biopsies from patients with chronic non-B hepatitis to determine if their hepatocytes contain HA Ag. Such studies are in progress.

(3) Non-A, Non-B, Hepatitis Viruses

Antigens

One approach to the identification of non-A, non-B hepatitis-associated antigens is the application of techniques useful in the detection and identification of HBV and HAV: immune electron microscopy, radioimmunoassays

and immunofluorescence. By application of radioimmunoassay techniques, we have identified a serum antigen associated with two cases of well-characterized non-A, non-B hepatitis. The appearance and disappearance of this antigen in the serum was temporally related to the hepatitis in these two individuals, and the antigen was shown to be particulate and biophysically characterizable by ultracentrifugation procedures. However, insufficient quantities of serum were available for more detailed analysis. Very preliminary antibody surveys suggested that antibody to this antigen was widespread among humans and chimpanzees and that, if it is an antigen associated with non-A non-B hepatitis, it is not associated with the major cause of this disease. Carriers of this antigen are being sought among populations known to have a high prevalence of HB_sAg carriage and HAV infection in order to collect sufficient material for large-scale purification and characterization.

Transmission of non-A non-B agents to chimpanzees

A number of unsuccessful attempts to transmit non-A non-B agents to primates were carried out in past years. However, recently two successful transmission studies were completed simultaneously, one by the Bureau of Biologics and the other by the Clinical Center Blood Bank in collaboration with our laboratory. In both studies the incubation period in chimpanzees was comparable to that for non-A non-B hepatitis in man, and both biochemical and histologic evidence of hepatitis was obtained. In our collaborative study, plasma or serum from patients with chronic non-A non-B hepatitis as well as from patients with acute infections transmitted disease to chimpanzees. Thus, proof of chronic carriage of non-A non-B viral hepatitis agents has been obtained.

In a collaborative confirmatory study, serial plasmapheresis units and serial liver biopsies were obtained from experimentally infected chimpanzees. Fluorescein-labeled convalescent serum from the original non-A non-B patients and from experimentally infected chimpanzees have been prepared and are being tested against acute phase frozen liver biopsies in an attempt to identify a viral antigen. In addition, the plasmapheresis units will be subjected to ultracentrifugal separations and studied by radioimmunoassay and immune electron microscopy techniques in a search for viral antigens. Also, previously infected chimpanzees are being cross-challenged and pools of infectious plasma are being identified and aliquoted for subsequent infectivity titrations. There is now a very high probability that the application of the many techniques developed for the study of HBV and HAV will rapidly lead to the identification and characterization of non-A non-B agents. The obvious first approach to control of non-A non-B hepatitis will be the development of tests for identifying blood donors capable of transmitting non-A non-B agents. Several approaches should be considered: It may be possible to identify a serum antigen that can be detected in a manner similar to HBs Ag. Alternatively, it may be possible to detect an antibody associated with recent or chronic non-A, non-B infection, such as anti-HBc in HBV infections. Finally, it might be possible to develop or identify some non-serologic test that has a strong positive correlation with presence of non-A non-B

agents. No matter what direction this research takes there will be a need for large quantities of viral antigen for the development of serologic tests, for the characterization of the agents and for seroepidemiologic studies. These will most likely come from large-volume purification of antigens from plasma of chronic carriers (one such known infectious carrier is already being plasmapheresed on a regular basis) or from extraction of antigens from liver tissue of experimentally infected chimpanzees in a manner similar to that used for production of hepatitis A viral antigen in marmosets.

Other

The development of tests for antigens and antibodies associated with non-A non-B hepatitis will provide the opportunity for seroepidemiologic studies similar to those carried out for types A and B hepatitis. These tests, plus the pools of virus that are being titered and certified for infectivity in chimpanzees will provide the means for attempts to isolate the virus(es) in vitro.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00027-11 LID

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
BASIC STUDIES OF MYCOPLASMAS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Joseph G. Tully, Ph.D. LID, NIAID Head, Mycoplasma Section

Others: David L. Rose LID, NIAID Research Microbiologist

COOPERATING UNITS (if any) State Univ. N.Y., Stonybrook, N.Y. (Dr. D.L. Williamson); Wistar Inst., Phila, Pa. (Dr. H.F. Clark); Yale Univ., New Haven, Conn. (Dr. A. Steere); Bureau of Biologics, FDS (Dr. M.F. Barile); U.S. Dept. Agriculture, Beltsville, Md. (Dr. R.F. Whitcomb and Dr. D. Stiller); Z01 AI 00007-4 LSD

LAB/BRANCH (Dr. R.M. Cole)
Laboratory of Infectious Diseases

SECTION
Mycoplasma Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These efforts cover both basic and applied aspects of mycoplasmas and related wall-free prokaryotes, including their neurotoxins, enzymes and other biological factors involved in virulence, their immunological interrelationships, and their possible role in human disease or in diseases of uncertain etiology. Current projects of interest concern the characterization of a new group of pathogenic spiroplasmas and sero-epidemiological studies on the human exposure to these new microbial forms. Development of an effective culture medium for the recovery and propagation of these organisms from host tissues has also provided a more effective medium for recovery of Mycoplasma pneumoniae from human tissues and washings, and for propagation of other fastidious mycoplasmas.

Project Description

Studies directed to various aspects of the biology and pathogenicity of helical mycoplasmas (spiroplasmas) have continued. Our characterization of these agents as the first known spiroplasmas with pathogenicity for vertebrates has opened up a number of areas for developing the possible role of these agents in human disease. Current work is directed to three broad areas, covering (1) the biological characterization of sprioplasmas, including theri serological and hiochemical interrelationships, (2) development of appropriate models to study their pathogenicity and host response, which includes not only small laboratory animals but primates, and (3) evaluation of various techniques (primary isolation in culture, serological, histological, etc.) to assess the possible role of spiroplasmas in acute and chronic human disease.

A. Cultivation of SMCA strains and biological properties

SMCA and GT-48 spiroplasmas were successfully grown on 2 of 20 different culture media preparations tried. Not growth occurred on standard mycoplasma media, nor on two other formulations that supported growth of other plant or insect spiroplasmas. Growth levels to 10^8 or 10^9 color-changing-units (or colong-forming-units on agar adapted strains) were observed in the most satisfactory culture medium (designated SP-4 medium). Terminal dilution procedures (greater than 10^{23}) were employed to exclude other possible agents in the cultures. The organisms were kept in continuous culture at both 30 and 37C, and were eventually purified by triple cloning procedures. While adaptation of strains to a simplier growth medium was successful, this medium was not adequate to recover the organisms directly from infected egg fluids. The organism was found to have a DNA G+C ratio of 29 moles % and a number of other biological properties (temperature requirements, nutritional needs, cell protein patterns on polyacrylamide gels, etc.) which distinguished it from other known spiroplasmas (Whitcomb, USDA).

B. Virus infection of SMCA

Both SMCA and GT-48 strains contained numerous virions characteristic of those we designated earlier as SV-C3 (spiroplasma virus-citri 3) and SV-C1, and found in various S. citri strains. C3 virus is a short-tailed, hexagonal virus with a tendency to emerge from infected spiroplasma cells in buds off of the membrane. At this point, C2 virus has not been observed in SMCA, offering some suggestion that SMCA may act as a useful indicator strain for plaque titrations of these viruses (Cole, LSD/NIAID).

C. Occurrence of other tick spiroplasmas and interrelationships

The 277 F spiroplasma, a third isolate from rabbit ticks, was recovered at the Rocky Mountain Laboratory in 1968 (J. Bacteriol. 95, 291, 1968) by Pickens and colleagues. It was initially thought to be a spirochete but has now been identified as a spiroplasma from work published on the plant and insect spiroplasmas. We have found this spiroplasma to be

serologically distinct from SMCA and GT-48 strains, avirulent for the chick embryo, and incapable of inducing cataracts in suckling rats. The organism grows well in SP-4 medium. The Maryland TP-2 strain is a recent (Fall 1977) isolate from rabbit ticks recovered in Beltsville, Md. by D. Stiller. The organism was recovered directly on SP-4 medium. This spiroplasma is serologically related to SMCA and GT-48 strains and distinct from 277F spiroplasmas. It has been shown in our Section to be virulent for the chick embryo and to induce cataracts in suckling rats, although the overall pathogenicity for vertebrates appears less than SMCA or GT-48 strains. (Whitcomb and Stiller, USDA).

D. Virulence of cultured SMCA for suckling rats and chick embryos

Cultured SMCA and GT-48 spiroplasmas produced cataracts and central nervous system disease in suckling rats and both strains were lethal for 7 day old chick embryos. These results were comparable to findings observed with egg-adapted lines of the agent. Strains grown at 30°C were also pathogenic for both animal hosts. The GT-48 strain was more virulent for the rat, with $\log_{10}$ LD_{50} values of 3.0 to 4.0 being observed. Egg LD_{50} values of 8.5 to 9.5 were obtained with cultured SMCA, which suggests that 1 helical organism is lethal for the chick embryo. SMCA cultures carried beyond 30 passages in artificial media showed some decline in virulence for suckling rats and in rate of cataract induction. The chick embryo seemed less suitable as a measure of virulence, since no decline in egg LD_{50} values was observed in tests with high-passage lines of the organism. In collaborative work at Wistar Institute, cultured SMCA has also been found to induce cataracts in baby rabbits. (Clark, Wistar Inst.).

E. Pathogenicity of SMCA for Primates

Preliminary challenge of a Cynomologous monkey with SMCA by intracerebral, intrathecal, and intravenous routes revealed some immediate CNS symptoms, including disorientation, incoordination, and some posterior paralysis. While these symptoms regressed over a 2 week period, there was some residual low level incoordination apparent 1 month post-challenge. A control monkey, receiving culture broth by the same routes, remained normal. Although A CF antibody response to SMCA occurred (32 fold increase in titer at 4 weeks), the high pre-challenge titer (1:128) was thought to have conditioned the observed response. Subsequently, about 1/3 of 20 normal Cynomologous monkeys were found to have pre-existing CF titers to SMCA (at levels of 1:32 to 1:64). Twenty sera from normal Rhesus monkeys did not show CF titers to SMCA. Additional attempts, although limited in number, to induce disease in Cynomologous monkeys free of SMCA antibody have yielded some evidence of short-term neurologic disease, primarily slight unilateral posterior paralysis that resolves in 1 month. CF antibody responses occur, sometimes reaching titers of 1:256 to 1:1280, but many animals show rapid declines in antibody response. Five monkeys with initial CNS responses are being held to assess long-term (1 year or more) consequence of the challenge (Barile, FDA).

F. Serological tests for spiroplasmas

Conventional mycoplasma serological tests appear less suitable for use with spiroplasmas, including those tests which can be used to assess serological relationships among individual strains or species and tests for use in seroepidemiology. Recent efforts have been directed to the spiroplasma "deformation" (DF) test for antigenic analysis of spiroplasma strains and some of the factors necessary to adapt the test to SMCA antigens. The DF procedure is based upon the activity of an antiserum to alter the helical structure of the organism. Specific hyperimmune rabbit serum to SMCA or GT-48 strains show DF titers of 1:16,000 to 1:32,000, with antisera to heterologous spiroplasmas showing DF titers of 1:20 or less. The test was found to work with antigens previously frozen and thawed (which has advantages in testing a number of different spiroplasma antigens), and the procedure has been modified to a microtiter plate technique. Complement is not required in the test and does not alter the final DF titers. The test is rapid (1-2 hrs for final reading), convenient, and specific for serological comparison of known spiroplasmas.

More recent attempts to improve serological study of spiroplasmas involves a combined "deformation/metabolic inhibition" (DF/MI) test. The MI procedure, a standard mycoplasma serological test, is based upon the ability of a specific antiserum to inhibit metabolism (glucose fermentation or arginine/urea hydrolysis) of organisms in broth cultures containing a pH indicator. The combined test involves reading DF titers from samples of the antigen-antibody mixtures of microtiter wells, resealing the microtiter plates, and reading the MI titers after 3-5 days of incubation. The results with SMCA antisera shows MI titers approximately 2-4 fold greater than DF titers, although some difficulty is experienced with clear cut carbohydrate fermentation with some SMCA antigen preparations. With some refinements, the combined test would seem to have advantages in versatility, depending upon available equipment for darkfield microscopy and the need for rapid test results, and possible application to measurement of spiroplasma antibody in human sera. (Williamson, State Univ. New York; Whitcomb, USDA).

G. Sero-epidemiology of SMCA in man

Serological screenings, using the CF test with SMCA antigen derived from broth-grown spiroplasmas, have now been performed on about 1000 sera from children and young adults. Paired sera of 169 normal children, in age groups less than 1 year to 12-13 years, showed an average 6.5% of individuals (11/169) had 4-fold or greater rises in CF antibody titer to SMCA antigen. No obvious increase in antibody rises were apparent with increasing age. On the other hand, paired sera from 105 children with various central nervous system diseases (mostly unclassified encephalitides or aseptic meningitis), and age matched with the normal group above, showed about twice the incidence of 4-fold or greater rises in SMCA CF titer (13/105 or 12%). (Differences between the two groups yielded p values of 0.1210 in the Fisher X^2 test.) CF titers of 1:4 to 1:8 against

SMCA antigen were observed in about 50% of the sera examined, regardless of the age group tested. One patient with Stevens-Johnson syndrome had a 16-fold increase in CF titer to SMCA, although at least two other children with this diagnosis did not exhibit antibody rises to the organism. A selection of sera from about 20 children with Reye's syndrome did not indicate any specific association with SMCA. These general observations seem to justify more extensive evaluation of the sero-epidemiology of spiroplasmas in man. (Chanock, LID; NIAID)

A number of unpaired sera from cases of Lyme arthritis observed at Yale University have been examined in the CF test with SMCA antigens. This epidemic form of arthritis has occurred frequently in eastern Connecticut in summer and early fall, occurs primarily in children, and is characterized by recurrent attacks of symmetric swelling and pain in a few large joints, especially the knee. No known viral or bacterial agent has been associated with the disease, although many of the cases have had a history of a tick bite. Serological tests on coded specimens possessed moderate CF titer to SMCA. The absence of acute phase sera or pre-illness sera prevents further interpretation of these results. Further attempts will be made to examine paired sera from cases of Lyme arthritis and for direct isolation of a possible spiroplasma from joint tissue. (Steere, Yale Univ.)

H. Other Mycoplasma Research

The SP-4 medium developed for SMCA spiroplasmas has made a major contribution to the cultivation of fastidious mycoplasmas. This medium, based upon conventional mycoplasma broth and tissue culture supplements, supports growth of a number of fastidious mycoplasmas (M. pneumoniae, and M. hyopneumoniae). We tested SP-4 medium on about 200 human throat specimens, previously tested and found negative for M. pneumoniae on conventional diphasic (agar-broth) media for this organism. Identification of agar colonies of M. pneumoniae on conventional diphasic media (with methylene blue) were 5/200 (5%); on SP-4 medium with methylene blue rates of 23/200 (15.3%) were observed; on SP-4 diphasic medium without methylene blue an isolation rate of 69/200 (34.5%) was recorded. Thus, SP-4 medium offered enhanced isolation of M. pneumoniae from so-called "negative" specimens and suggests that some important mycoplasma pathogens can be missed from deficient culture procedures.

A number of other projects have been recently completed or are in progress within the Section. These include:

a) studies on the binding of plant lectins to mycoplasma membranes, which are designed to understand exposed chemical groups on cell membranes and their possible role in mycoplasmas attachment to cells.

b) the occurrence of certain sugar transport enzymes in mycoplasmas that do not specifically exhibit clear evidence of carbohydrate fermentation or other energy-yielding mechanisms.

c) the ability of certain mycoplasmas to induce lymphocyte transformation and the role of specific components of mycoplasmas in mitogenic activity.

d) taxonomic characterization of new mycoplasmas from animals in the wild, in an attempt to understand how man interacts with all mycoplasmas in his environment.

e) DNA hybridization tests on a number of unclassified sterol nonrequiring mycoplasmas (acholeplasmas), organisms that occur frequently in a large number of animal hosts and in cell culture contamination.

f) the use of the SP-4 medium to study the occurrence of M. pneumoniae in children under the age of 5--information that would help answer the question why low isolation rates of this organism are recorded in children who possess mycoplasmacidal antibody.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00028-20 LID

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

STUDY OF RESPIRATORY VIRUSES AND MYCOPLASMAS IN VOLUNTEERS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Brian R. Murphy, M.D.	NIAID, LID	Medical Officer
	Robert M. Chanock, M.D.	NIAID, LID	Chief, LID, NIAID
	Lewis J. Markoff, M.D.	NIAID, LID	Medical Officer

COOPERATING UNITS (if any)

Flow Laboratories, Rockville, Md.; University of Maryland School of Medicine, Baltimore, Md.; Children's Hospital National Medical Center, Washington, D.C.; University of Rochester, Rochester, N.Y.; Vanderbilt University, Nashville,Tenn. Baylor College of Medicine, Houston Tx.

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
36/12	12/12	24/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We evaluated the response of adult volunteers to a series of ts or cold adapted recombinants of influenza A virus. A/Victoria/75 ts-1[E] recombinants with a 38°C shutoff temperature and ts lesions in RNA segments 1 and 5, retained some virulence for individuals who lacked immunity to the hemagglutinin and neuraminidase antigens of the virus. In contrast, a more temperature sensitive A/Victoria/75 ts-1A2 recombinant, with a 37°C shutoff temperature and ts lesions in RNA segments 1 and 2, was satisfactorily attenuated for doubly seronegative volunteers. Contrasting responses were observed with 2 ca recombinants; A/Scotland/74 ca retained some virulence, while A/Victoria/75 ca was satisfactorily attenuated.

Project Description

Objectives: (1) To evaluate temperature-sensitive mutants and recombinants of influenza A virus for evidence of attenuation and antigenicity in volunteers; (2) to characterize the serum and local respiratory tract anti-neuraminidase and anti-hemagglutinin antibody responses of man to infection with wild type or attenuated influenza A virus; (3) to evaluate the infectivity and attenuation of ts mutants of M. pneumoniae.

Major Findings

Evaluation of Immunity to the Neuraminidase Antigen and its Role in Attenuation

A/Vic/75-ts-1[E] recombinant clones 81 and 113 both bore the $H3_{75}N2_{75}$ surface antigens and retained some residual virulence for adults who lacked immunity to the $N2_{75}$ neuraminidase, as well as to the $H3_{75}$ hemagglutinin. Since lack of neuraminidase immunity was suggested as being a contributing factor to the reactogenicity of earlier ts-1[E] viruses in children, we decided to evaluate the importance of neuraminidase immunity in the reactogenicity of Vic/75 ts-1[E] recombinants. We isolated an A/Vic/75-ts-1[E] virus (clone 67) that bore the two ts-1[E] genes and the neuraminidase gene of HK/68-ts-1[E] ($N2_{65}$). This virus was evaluated in a group of young adults who lacked antibody to $H3_{75}$ and to $N2_{75}$ but who had moderate to high levels of anti-$N2_{65}$ antibody. Clone 67 was considerably more attenuated than the Vic/75-ts-1[E] clone 81 and 113 recombinants that bore the $N2_{75}$ surface antigen; fever and/or systemic illness was observed in less than 5% of volunteers given the clone 67 whereas clones 81 and 113 both induced febrile or systemic illness in approximately 12% of vaccinees.

Vaccinees who received the clone 67 developed an anti-$H3_{75}$ response but not an anti-$N2_{75}$ response. By challenging such volunteers with Vic/75 ($H3_{75}$ $N2_{75}$) wild type virus it was possible to assess the contribution of anti-hemagglutinin immunity to resistance to influenza A virus in the absence of neuramindase immunity. Each of the vaccinees who developed a seroresponse to the $H3_{75}$ antigen following infection with clone 67 was protected against illness but not infection when challenged with wild type $H3_{75}$ $N2_{75}$ virus. This indicates that hemagglutinin-specific immunity, like neuraminidase specific immunity, can protect against illness without preventing infection. Formerly it had been thought that this was only a property of neuraminidase specific immunity.

Evaluation of A/Vic/75-ts-1A2 Recombinant Viruses

When immunity to both surface antigens of influenza A virus is lacking, as at the time of a pandemic shift or as occurs in infants, only the degree of defectiveness of the vaccine virus determines attenuation and in this situation defectiveness must be greater than that specified by the ts-1[E] virus. It was possible to produce a recombinant with a set

of ts lesions that specified a greater degree of defectiveness than was seen with the ts-1[E] recombinants. This recombinant, designated Vic/75-ts-1A2, had a 37°C shutoff temperature and was from 100 to 1000 fold more restricted in efficiency of plaque formation at 37°C than the Vic/75-ts-1[E] virus.

An A/Victoria/75 ts-1A2 recombinant, that possessed ts mutations on the genes coding for the P1 and P3 proteins and that was markedly restricted in its replication at 37°C, was administered to 42 doubly seronegative volunteers at a dose of $10^{7.5}$ $TCID_{50}$. Prior to this trial we were concerned that the 1A2 recombinant might be overattenuated. Previously the A/Hong Kong/68 ts-1[A] recombinant, that had a 37°C shutoff temperature for plaque formation and ts mutations affecting the same proteins as the 1A2 recombinant, was found to lack infectivity for adults who had neuraminidase immunity but lacked serum HI antibody. Nonetheless, the A/Victoria/75 ts-1A2 recombinant infected 90% of the volunteers. Only 5% of the volunteers developed mild coryza or rhinitis; other signs or symptoms were not observed. This level of reactogenicity was considered completely acceptable. Vaccinees shed virus for a shorter interval and at a lower titer than the Vic/75-ts-1[E] vaccinees. Each isolate retained the ts phenotype indicating that the recombinant was stable genetically in doubly seronegative adults. Eighty six percent of the vaccinees developed a rise in serum HI antibody. These preliminary clinical data are extremely encouraging since they indicate that the A/Vic/75 ts-1A2 recombinant was more attenuated than the A/Vic/75 ts-1[E] recombinants. Further, the 1A2 recombinant was surprisingly antigenic considering its limited degree of replication. Finally, the ts-1A2 recombinant induced significant resistance to subsequent challenge with A/Vic/75 wild type virus.

Evaluation of cold-adapted influenza A recombinants in adults - Adult seronegative volunteers were administered $10^{8.5}$ or $10^{7.5}$ $TCID_{50}$ of A/Scotland/75 (CR18, clone 7) or $10^{7.5}$ $TCID_{50}$ of A/Vic/75 (CR19) cold-adapted recombinants to assess the level of attenuation and antigenicity of these candidate live influenza A virus vaccines. In tissue culture these 2 viruses had a shutoff temperature for plaque formation of 39°C unlike their cold-adapted parent (PI-7) which had a shutoff temperature of 37°C. The CR18 recombinant was given to 12 seronegative volunteers at a dose of $10^{8.5}$; 4 of the volunteers developed a febrile or systemic reaction. At a dose of $10^{7.5}$ 1 of 8 infected volunteers developed a febrile response (39°C). Thirteen volunteers received the CR19 vaccine; one developed a mild systemic reaction associated with rhinitis; two others had upper respiratory tract symptoms. Infected volunteers shed virus in low titer for a short period of time. The vaccine strains were as effective as the wild type parent in inducing a serum HI antibody response. The reactogenicity of these recombinants may be a result of their high shutoff temperature or of the absence of hypothetical non-ts, non-ca attenuating genes transferred from the ca parent. In any case, these observations suggest that the ca phenotype of a recombinant influenza A virus may not always specify a satisfactory level of attenuation for man.

Virus given to volunteers was prepared and safety tested by Flow Laboratories, Rockville, Maryland (Contract # NO1 AI 32521).

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00029-04 LID

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

STUDIES ON TS MUTANTS OF STREPTOCOCCUS PNEUMONIAE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Michael B. Grizzard	LID, NIAID	Res. Assoc.
	Dr. Robert M. Chanock	LID, NIAID	Chief

COOPERATING UNITS (if any)

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LAB/BRANCH

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SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is to be terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00175-01 LID

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

LABORATORY STUDIES OF PARAMYXOVIRUSES AND RESPIRATORY SYNCYTIAL VIRUS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert M. Chanock	LID, NIAID	Chief
	Linda S. Richardson	LID, NIAID	Sr. Staff Fellow
	Robert B. Belshe	LID, NIAID	Medical Officer
	Gregory A. Prince	LID, NIAID	Sr. Staff Fellow

COOPERATING UNITS (if any)

W.T. London (NINCDS); Molecular Anatomy Program (Dr. J. Gerin)

LAB/BRANCH

Laboratory of Infectious Diseases

SECTION

Respiratory Viruses Section

INSTITUTE AND LOCATION

National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
66/12	30/12	36/12

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SUMMARY OF WORK (200 words or less - underline keywords)

Respiratory syncytial (RS) virus ts mutants derived in LID were compared genetically with ts mutants produced in Glascow and a total of 5 complementation groups were identified. Characterization of virus shed by chimpanzees infected with the ts-1, ts-1 NG-1, ts-1 NG-16, ts-2 or ts-7 mutant indicated that none of these viruses was completely stable genetically. However, evaluation of apparent ts+ "revertants" in primates indicated that these viruses (that had regained the capacity to produce plaques efficiently at restrictive temperature) were attenuated. It is likely that modifying mutations developed rather that true reversion.

Experimental RS virus infection of the cotton rat was studied in detail and many similarities to human infection were noted. The cotton rat develops histopathologic evidence of disease in the nasal turbinate, bronchial and bronchiolar epithelium. This experimental rodent model of RS infection should prove useful in elucidating pathogenesis of disease.

This project was previously a part of Project # Z01 AI 00024-18 LID

Project Description

This year the respiratory syncytial virus program gave special emphasis to 4 main areas of research: 1) comparison of the in vitro properties of the NIH ts mutants with those of the Glascow/ts mutants; 2) development of animal models of RS virus infection in which to study viral growth and pathogenesis of disease; 3) evaluation in experimental animals of ts mutants that appeared to be promising vaccine candidates; 4) development of an ELISA for detection of RS virus antibodies.

Major Findings

Genetics of Respiratory Syncytial Virus

Background - RS virus is the major etiologic agent of bronchiolitis and pneumonia of early life and the need for effective immunoprophylaxis has been clearly established in studies performed throughout the world. This virus has a non-segmented genome and does not undergo genetic reassortment. Initially a series of 7 ts mutants of RS virus were produced by chemical mutagenesis with the expectation that their temperature sensitivity would restrict growth in the target organ, the lungs.

Complementation Analysis of Additional ts Mutants of RS Virus - Based on the probable molecular weight of the RS virus genome, the existance of at least 6-7 complementation groups is likely. In this laboratory ts mutants were grouped previously into 3 complementation groups based on their ability to complement one another at the restrictive temperature of 38° or 39°C. Six ts mutants of RS virus isolated in the laboratory of Dr. Craig Pringle (Glasgow, Scotland) were tested in our laboratory by complementation using the prototypes from the NIH and Glascow complementation groups. On the basis of this analysis, we were tentatively able to identify 2 new complementation groups of RS virus. Three of Pringle's ts mutants can be grouped into our Group A, one mutant in a new Group D and two mutants in a new Group E. Temperature shift experiments with the new Group A mutants of Pringle confirm that the ts lesion of members of complementation group A affects a late function that is required for at least 10-13 hours in the replicative cycle. Further characterization of these mutants is in progress.

In terms of future directions it is now clear that the ts-2 mutant, a member of complementation group B, is the most attenuated of the mutants thus far tested. This mutant, that is defective in adsorption and penetration of cells, has reduced infectivity for the chimpanzee and is completely attenuated for this host. The gene that is the locus of the ts-2 mutation offers a desirable target for further attempts at mutagenesis if the ts-2 mutant does not prove to be acceptable as a vaccine strain for infants and young children.

Experimental Immunoprophylaxis Against Respiratory Syncytial Virus Infectious In Primates

Background - Last year 5 ts mutants of RS virus were evaluated for virulence in seronegative chimpanzees. Two of the mutants, ts-1 and ts-7, induced rhinitis similar to that seen during infection with wild type virus. The more defective derivatives of ts-1 into which additional mutations were introduced, i.e., ts-1 NG-1 and ts-1 NG-16, induced very mild rhinitis which was less extensive than that observed during infection with wild type virus, ts-1 or ts-7. Significantly, the ts-2 mutant was less infectious for chimpanzees than the preceding viruses and infection, when it occurred, was not accompanied by signs of respiratory tract disease.

Genetic Stability of ts Mutants in Chimpanzees - Viruses shed by chimpanzees infected with the various ts mutants of RS virus were analyzed for ts phenotype by assaying efficiency of plaque formation at permissive and restrictive temperatures. None of the 5 mutants tested (ts-1, ts-1 NG-1, ts-1 NG-16, ts-2 and ts-7) was completely stable genetically during replication in chimpanzees. A similar temporal pattern was seen for genetically altered virus produced during infection with ts-1, ts-1 NG-1, ts-1 NG-16 or ts-7. For the first one to six days the virus shed was entirely ts. There followed a period of 3 to 8 days during which genetically altered virus was shed continuously. In some instances (ts-1 NG-1, ts-1 NG-16 and ts-7) genetically altered virus was shed throughout the late course of infection; but in other instances (ts-1 and one animal infected with ts-1 NG-1) the genetically altered virus disappeared, and only ts virus was shed late in infection. A different pattern was seen in the ts-2 infected chimpanzees; three animals shed only ts_+ virus throughout infection, while the remaining 2 animals shed only ts^+ virus which was indistinguishable from wild type in its efficiency of plaque formation at restrictive temperatures.

The nature of the genetically altered virus shed from the upper respiratory tract was also evaluated by testing clonal populations (obtained directly from nasopharyngeal swab samples) for their ability to form plaques at restrictive temperatures. In this manner mixtures of ts phenotypes could be identified. The major proportion of virus shed by the ts-1 and ts-1 NG-1 infected animals retained the ts phenotype. This was not the case for certain chimpanzees infected with the ts-1 NG-16, ts-7 or ts-2 mutant; in these instances the majority of virus shed had lost some or all of its temperature sensitivity. One ts-1 infected chimpanzee shed ts, intermediate and ts^+ (wild type) viruses, while all of the virus from two ts-2 infected chimpanzees was ts^+. A ts-1 NG-16 infected animal shed ts virus and virus intermediate with respect to temperature sensitivity of plaque forming ability. The ts-7 infected animal shed both intermediate and ts^+ viruses.

Two clones of "revertant" ts-2 were evaluated in chimpanzees to determine whether virulence had been restored. Chimpanzees shed these viruses in

as high a titer as wild type RS virus. Significantly, however, the chimpanzees infected with "revertant" ts-2 failed to develop any signs of upper respiratory tract disease. This observation was confirmed when 4 other clones of "revertant" ts-2 were evaluated in owl monkeys. None of the owl monkeys developed rhinorrhea although each animal became infected. These primates shed considerably less virus than monkeys infected with wild type virus. The "revertant" ts-2 virus was more infectious than its parent since the 2 chimpanzees and 8 owl monkeys became infected when given 20 to 40 fold less virus than was required to infect these primates with the parental ts-2 mutant.

The observations made during these studies stress the importance of evaluating candidate live vaccine viruses in vivo, since none of these viruses was completely stable genetically in chimpanzees. However, the genetically altered viruses produced by chimpanzees infected with either derivative of ts-1, i.e., ts-1 NG-1 or ts-1 NG-16, retained a degree of temperature sensitivity with the exception of one isolate of ts-1 NG-16. Of particular interest was the finding of "revertant" ts-2 (ts^+ in phenotype) shed by two of the five chimpanzees infected with the ts-2 mutant. Although the "revertants" were indistinguishable from wild type RS virus in high efficiency of plaque formation at 39°C and syncytium forming ability, none of the six "revertant" clones was virulent when evaluated in chimpanzees or owl monkeys. These findings suggest that true reversion by back mutation did not occur. The most likely explanation is that a modifying mutation occurred that restored the ts^+ phenotype and syncytium forming ability without restoring virulence.

Genetic stability of the ts-2 mutant in the chimpanzee is similar to that recorded for the attenuated poliovirus vaccine strains in man. "Revertant" virus, which has lost some or all of the temperature sensitivity of the vaccine virus (i.e., loss of the rct marker) or which has regained some neurovirulence for the monkey, is frequently recovered from the stools of vaccinees infected with attenuated poliovirus. However, such altered virus is rarely associated with paralytic disease in either vaccinees or contacts of vaccinees; those rare individuals who develop paralytic disease are often immunodeficient. Indeed, it appears that vaccine virus or genetically altered vaccine virus which has lost some of its temperature sensitivity has replaced wild type poliovirus as the predominant virus circulating in the United Kingdom. Predominance of these viruses, however, is not associated with appreciable paralytic disease, suggesting that transmission of vaccine virus or partially altered vaccine virus may extend the benefits of immunization without significant risk to the community. ts-2 "revertants" may well behave in a similar manner. For this reason and because of its complete attenuation for both chimpanzees and owl monkeys, the ts-2 mutant should be evaluated in children for safety and for its usefulness in preventing RS virus disease in infancy and early childhood.

Bovine RS Virus - The bovine respiratory syncytial (BRS) virus was also evaluated in seronegative chimpanzees. This virus is restricted in

growth in human and simian cells although in bovine cells (BEK) it grows as well as human RS virus. Furthermore, antibody to BRS virus neutralizes human RS virus efficiently in the plaque reduction assay and BRS infected cells are stained by fluorescein tagged antiserum containing antibodies directed against the human RS virus. Two chimpanzees were inoculated intranasally with $10^{7.0}$ $TCID_{50}$ of BRS virus. Virus was recovered from only one of the two animals and the quantity of virus shed was very small (10 $TCID_{50}$/ml of nasopharyngeal swab sample). The chimpanzees did not become ill nor did they develop neutralizing antibody to the human RS virus. These animals were not resistant to subsequent challenge with the human RS virus. We are presently adapting BRS virus to grow in HEK cells in an attempt to select a mutant of BRS virus which will grow moderately well in primate cells. A mutant which is partially restricted in primate cells should be attenuated for primates and thus might be suitable for use in infants for prevention of serious RS virus disease.

Experimental Infection with Respiratory Syncytial (RS) Virus in Cotton Rats

Background - Experimental study of the basic mechanisms of respiratory syncytial (RS) virus disease, and of the factors mediating recovery and immunity has been hampered by the lack of an appropriate small animal in which to investigate these questions. The recent description of RS virus infection in infant ferrets provided the first such model. However, the striking age dependence seen in pulmonary RS virus infection in ferrets, whereby infant animals rapidly become refractory to RS virus replication in the lung, disqualifies this model for extended studies of RS virus infection in the lower respiratory tract.

In searching for an alternate experimental animal which would support RS virus replication in the lung at all ages, we found the cotton rat (Sigmodon hispidus) to be suitable. We have subsequently studied the pathogenesis and immunology of RS virus infection in these animals.

Pattern of Infection in Cotton Rats - RS virus infection of cotton rats is an acute infection involving both upper and lower portions of the respiratory tract. Virus replication, as monitored by immunofluorescence microscopy, is restricted to the respiratory epithelium. There is an excellent correlation between histologic findings, immunofluorescent observations and viral replication in each of the three portions of the resiratory tract examined. Virus grows to high titer in nasal turbinates, infects a majority of epithelial cells and produces a moderately severe desquamative, exudative rhinitis. The trachea is relatively resistant to infection, virus grows to a low titer and does not induce any histologic changes. The lungs, like the nose, support viral replication to high titer. Infection, as monitored by immunofluorescence microscopy, is restricted to bronchial and bronchiolar epithelium and is associated with mild bronchitis and bronchiolitis.

Experimental RS virus infection in cotton rats bears several similarities to natural RS virus infection in man. First, rhinitis is a prominant pathologic response to infection in the cotton rat. Similarly, rhinitis is a common clinical response observed during human infection. The restriction of viral replication to epithelial cells also agrees with the very limited data available from immunofluorescence studies of specimens from infants with RS virus disease. The time course of infection in man and cotton rats is also similar. Finally, the pulmonary component of RS virus infection in cotton rats resembles that of the severely affected young infant with RS virus disease. It should be noted, however, that bronchiolitis in the latter instance may be quite extensive, while in the cotton rat bronchiolar changes are considerably less severe.

Comparison of the cotton rat model with the infant ferret model indicates some similarities, and some important differences. Both animals undergo a mild, acute respiratory tract infection, with both the upper and lower portions of the respiratory tract involved. Pattern of viral replication in the nose is similar in both animals, as are histologic findings (although pathologic changes are somewhat more pronounced in the cotton rat). While both the infant ferret and cotton rat support viral growth in the lungs, the pattern of infection differs markedly. Viral antigen in the ferret lung is almost exclusively restricted to the alveolar cells, with only rare, single-cell involvement of bronchiolar epithelium. The opposite is true in the cotton rat, where viral antigen is seen only in bronchial and bronchiolar epithelium, with only rare, isolated involvement of alveolar cells. We have no current explanation for this difference.

Another striking difference between the ferret and cotton rat is the extent of the serum antibody response. Ferrets infected as early as the day of birth or as late as 28 days of age develop little or no serum neutralizing antibody. In contrast, cotton rats infected shortly after birth develop neutralizing antibody, in some instances to extremely high titer, by the ninth day after infection. Antibody response is not influenced by age at the time of infection. When ferrets and cotton rats are infected at three days of age both support RS virus replication in the lungs and nose to the same level and for the same interval. However, the antibody response of the rat is 100-fold or more greater than that of the ferret.

The most significant difference between the two experimental models of infection involves the effect of age upon susceptibility of the lungs to virus and the level of viral replication in this site. Whereas the lungs of 28 day old ferrets are completely refractory to RS virus infection, the lungs of cotton rats are uniformly susceptible to infection throughout life. Continuous susceptibility of the cotton rats' lungs offers several possibilities to explore aspects of RS virus pathogenesis previously not amenable to experimental study. First, the continuous susceptibility of the lungs to RS virus infection makes it possible to study the effect of vaccination upon resistance to infection. Use of

this animal model might also elucidate the mechanism by which formalin-inactivated, alum-precipitated RS virus vaccine potentiated, rather than protected against disease in young children. Second, this model should permit an experimental study of the persistence of immunity following infection. In man it is clear that immunity to RS virus infection is transient and incomplete, but the reasons are not well defined.

Other characteristics of the cotton rat also serve to enhance its desirability as an experimental model. In contrast to ferrets, which require rather careful attention to housing and breeding conditions, cotton rats do not need special caging, do very well on standard rat chow without supplements, require no special lighting cycles, and breed spontaneously throughout the year. This last characteristic offers the possibility of a detailed study of maternal-infant transfer of resistance, and the effect of these immunologic factors on RS virus infection.

Duration of Immunity - Intranasal infection of infant or adult cotton rats confers complete immunity to RS virus in both the upper and lower components of the respiratory tract for at least 4 months, the maximum time interval examined thus far. This study will continue until the duration of immunity is determined. Then, post-immune and immune animals will be compared to determine the factors governing immunity.

Transfer of Immunity from Mother to Infant Cotton Rat - Previous studies in ferrets demonstrated that dams infected with RS virus during pregnancy endowed their offspring with significant resistance to RS virus infection. Similar studies in cotton rats have confirmed these observations in the ferret. Infant cotton rats, born to mothers infected three to four weeks previously with RS virus, are completely resistant to RS virus replication in the lungs, and are completely or almost completely resistant to growth of virus in the nose. This protection is transient, and by seven weeks of age infants no longer have serum neutralizing antibody directed against RS virus, and are as susceptible as control animals to both nasal and pulmonary infection. The mechanisms of passively transferred immunity in the cotton rat have yet to be defined. However, preliminary data suggest that serum antibody does not mediate protection. Infants of mothers infected three to four weeks antipartum are markedly resistant to RS virus infection. These animals have serum neutralizing antibody titers of 1:100 to 1:400 at birth. Infants of the same age, but born to mothers infected four months earlier, have equivalent levels of serum neutralizing antibody, but are not resistant to RS virus infection. Yet in both cases, the mothers are completely immune to reinfection. This finding agrees with observations made in human infants which suggest that passively acquired serum antibody does not confer immunity to RS virus. Further studies are in progress to determine the factors governing passive immunity. Foster nursing experiments should permit us to determine whether parental transfer of immunologic factors across the placenta, or postnatal transfer through the colostrum and milk are responsible for protection. These will be followed by fractionation experiments to pinpoint protective factors. Our present operating hypothesis envisions

transfer of RS virus sensitized, immunocompetant cells from the mother via colostrum to the infant with population of the respiratory tract submucosal area providing a basis for temporary resistance to infection. Experimental studies in mice by Billingham and colleagues indicate that transfer of immunocytes from mother to infant mouse occurs. Furthermore, breast feeding during the first month of life appears to protect human infants from RS virus disease during infancy and early childhood. Studies in cotton rats may permit an elucidation of this phenomenon.

Immunization by Parenteral Inoculation of Live RS Virus - Scientists at Merck (West Point, Pa.) have tested a live RS virus vaccine which was administered parenterally to young children. The Merck vaccine preparation contained a low concentration of live virus (10^3 pfu/ml) but nonetheless induced a serum antibody response in most young seronegative vaccinees. Unfortunately, the basis for this immunologic response was not defined by the Merck workers before the vaccine was given to young children. Specifically, it was not established whether the live virus administered parenterally replicated or induced its effect through preformed non-replicating antigen. This is an important issue since an inactivated RS virus vaccine tested previously in infants and young children potentiated disease when the vaccinees underwent natural infection with the virus. Hence the urgency of elucidating the manner in which parenterally administered live RS virus induced an immunologic response.

A single intramuscular (IM) injection of wild type RS virus (10^4 plaque forming units) induced complete resistance to virus in both the nose and the lungs of cotton rats challenged intransally 3 weeks later with homologous virus. IM immunization stimulated a high level of serum neutralizing antibody which was of the same order of magnitude as that seen in convalescent animals infected initially via the intranasal route. A lower dose (10^2 pfu) of live virus contained in a vaccine prepared by Merck also induced substantial protection. A single dose of Merck vaccine induced nearly complete protection in the lungs but the nose was less well protected. Two doses, separated by two weeks, induced resistance equivalent to that stimulated by the higher dose of wild type virus.

The possibility that viral replication is necessary for inducing an immunologic response and resistance was examined in two ways. First, animals inoculated intramuscularly with live virus were sacrificed at intervals up to a week after inoculation, and various tissues, including the injection site, heart, lung and nose were examined for infectious virus. In addition, muscle from the injection site was sectioned with a cyrostat and examined for the presence of viral antigen by immunofluorescence. Neither of these approaches yielded evidence indicating that viral replication had occurred in any of the tissues examined.

Next, virus was inactivated by formalin or ultraviolet radiation, to determine if inactivated virus was as effective as live virus in stimulating immunity. Similar results were obtained with both methods of inactivation.

The serum antibody response of cotton rats receiving inactivated virus, and the resistance of these animals to intranasal challenge with RS virus were greatly reduced compared to animals which were given an equivalent suspension containing live virus.

Failure to detect viral replication at the infection site or at a distant site suggests that growth of virus is not essential for successful immunization. However, replication may occur at a level below the sensitivity of our assay systems. Study of inactivated virus on the other hand, suggests that replication is necessary for successful immunoprophylaxis, as two different methods of inactivation reduced or ablated an effective immune response and the induction of resistance to challenge. It is possible, however, that both formalin and ultraviolet light alter the critical antigen involved in resistance, and that an alternate method of inactivation which spares this antigen, might yield an effective immunogen.

Finally, studies are currently in progress to examine the effect of intramuscular administration of live virus to cotton rats possessing passive antibody received from their mothers. The individuals most in need of an effective RS virus vaccine are young infants, most of whom possess serum antibody passively acquired from their mother. Hence it is essential to determine whether parenterally administered live virus can stimulate effective immunity in the presence of varying levels of passively acquired serum antibody.

Relationship of Bovine and Human RS Viruses - The possibility that bovine respiratory syncytial (BRS) virus, a related though heterologous virus, could confer protection against human RS virus infection was examined. Animals which were administered BRS virus intranasally or intramuscularly developed complete resistance to subsequent nasal challenge with the human RS virus. The pathogenesis of BRS virus infection in cotton rats is now under investigation.

Development of An Improved Serologic Technique for Measurement of Immunologic Response to Respiratory Syncytial Virus

Background - The immunolgic response to respiratory syncytial (RS) virus infection has been measured in the past by the detection of a rise in complement fixation (CF) antibodies in the convalescent serum of the infected individual. Failure to detect a CF antibody rise in young infants during convalescence from severe respiratory disease caused by RS virus has variously been ascribed to immunologic immaturity of the infant or to the presence of preexisting maternal antibodies which mask an immune response or impair the ability of the infant to mount an immunologic response. More recently, the plaque reduction test has been used for assaying RS neutralizing antibodies in human sera. This technique is more sensitive than the CF test, however, the difficulty of performing the plaque assay as well as the longer time necessary to

carry out the test has limited its general use. We have therefore developed a sensitive and rapid assay for serum antibodies to RS virus utilizing the enzyme-linked immunosorbent assay (ELISA) technique that should have wide applicability in diagnostic and seroepidemiologic investigations.

ELISA for RS Virus Antibodies - The ELISA technique for antibodies to RS virus utilizes HEp-2 or African green monkey kidney (AGMK) grown RS virus as antigen. A dilution of the frozen and thawed virus-cell suspension is used without further manipulation in the ELISA test in order to provide both internal and surface viral antigens for interaction with antibodies. Alkaline phosphatase conjugated to anti-human IgG serves as the enzyme-linked antiglobulin.

Acute and convalescent phase sera from 91 infants and children hospitalized with RS virus lower respiratory tract disease were assayed by the CF, plaque reduction and ELISA techniques to define the relative efficiency of the three methods. Previously the diagnosis of RS virus disease had been established by virus isolation and/or a 4 fold or greater rise in serum CF antibody titer. The ELISA test proved more sensitive than CF for detecting RS virus serum antibody since 40 of 55 acute phase sera with a CF antibody titer of 1:4 had RS antibody activity at a serum dilution of 1:100 or greater in the ELISA test. In no instance was complement fixing antibody detected without concommitant ELISA antibody.

In general there was concordance between the plaque reduction and ELISA techniques for detection of RS serum antibodies although the latter method usually yielded a higher titer. Fifteen of the acute phase sera which lacked detectable plaque reduction antibody had antibody demonstrable by ELISA. In no instance was plaque reduction antibody activity present without corresponding ELISA antibody activity.

The efficiency of the CF, plaque reduction, and ELISA techniques for serodiagnosis was compared using 91 paired sera from infants and children with RS virus disease of the lower respiratory tract. In the 1-3 month old age group RS virus was isolated from the oropharynx of each of the patients, yet only 1 of 49 developed a significant serum CF antibody rise to RS virus. Also only one of 47 young infants had a 4-fold or greater rise in plaque reduction antibody. In contrast to the results obtained with these techniques, 25 of the 49 infants had a significant antibody rise when tested by ELISA, indicating the greater efficiency of the ELISA technique for detection of an RS virus serologic response during early infancy, the period when this virus is most important as a cause of serious lower respiratory tract disease. This greater efficiency of the ELISA technique was also evident to a lesser degree in the 4 to 6 month age group. In infants over 7 months old, the number of significant antibody rises detected by each of the 3 methods was similar.

The ELISA technique appears to be a specific and sensitive method for detection of RS virus serum antibodies. The test has the advantage of

measuring higher titers of antibody than the CF and plaque reduction techniques, the current standard methods of assaying serum antibodies to RS virus. In addition, the ELISA appeared to be more efficient than the plaque reduction and complement-fixation techniques for detecting a serologic response in young infants (1 to 6 months old) who are the major target for serious RS virus lower respiratory disease. The simplicity, sensitivity, and rapidity of the ELISA system make it a useful tool for immunologic studies with RS virus.

We are currently attempting to apply the ELISA to measure local secretory IgA response to RS virus infections. Since local immunity is known to play an important role in resistance to RS virus disease, and the current method (plaque reduction assay) of measuring IgA antibodies to RS virus is less than satisfactory, this is clearly an area of research that needs to be developed.

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LABORATORY OF VIRAL DISEASES
1978 Annual Report
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PHS-NIH
SUMMARY REPORT

ANNUAL REPORT OF THE LABORATORY OF VIRAL DISEASES, NIAID
October 1, 1977 - September 30, 1978

Dr. Wallace P. Rowe
Chief, Laboratory of Viral Diseases

The past year has been a very mixed one for the Laboratory of Viral Diseases. Administratively and personally it has been an extremely difficult year, with many stresses, tensions, and distractions. Scientifically, LVD continues to do quite well and can report a number of solid accomplishments.

With regard to the difficulties within LVD, these stem in large part from the laboratory chief's involvement in recombinant DNA committee and risk assessment work. As with the preceeding year, the urgency of the recombinant DNA crisis for NIH and for biomedical research in general has made immense demands on the lab chief's time. Along with Dr. Malcolm Martin of NIAID, the laboratory chief has spent the majority of his time in such activities as editing and preparing the report of the Falmouth, Massachusetts Workshop on "Risk Assessment of Recombinant DNA Experimentation with Escherichia Coli K12"; doing a large part of the organizing and writing up of the reports of the "U.S.-EMBO Workshop to Assess the Containment Requirements for Recombinant DNA Experiments Involving the Genomes of Animal, Plant and Insect Viruses" held in Ascot, England, and the "Virus Working Group Meeting" chaired by Dr. Harold Ginsberg; planning the risk assessment experiments in the P4 facilities at Ft. Detrick and NIH; participating in the meetings of the NIH Recombinant DNA Advisory Committee, the NIH Recombinant DNA Executive Committee, and Dr. Fredrickson's staff meetings on recombinant DNA policy matters. A result of this demand on the lab chief's time has been a serious breakdown in communication within LVD and the inability of the lab chief to devote the time necessary to give proper leadership to the LVD as a whole. This pressure, combined with a serious difference of opinion between the laboratory chief and Dr. Lewis over the appropriateness of a paper for publication, led the laboratory chief to request that a reorganization be instituted whereby the portions of LVD that are most remote from the lab chief's area, both physically and scientifically, be removed from his administrative responsibility. Dr. Lewis has been transferred from LVD to the Office of the Scientific Director and a number of other reassignments are under consideration at the present time. It is the hope of the laboratory chief that LVD can be consolidated into a more programmatically homogeneous laboratory and one in which the constituent units are located in reasonable physical proximity.

On the positive side, the scientific program of LVD remains strong, particularly in the area of oncogenic viruses. The murine leukemia virus program has made much progress in the study of the "MCF" viruses. These viruses, which were discovered in LVD last year, are recombinants between different endogenous viruses of the mouse, and are of major importance in the biology of virus-associated spontaneous neoplasms of the mouse. The involvement of Dr. Rowe with recombinant DNA activities and the risk assessment experiments has had some very positive scientific byproducts for LVD, in that the successful cloning

of polyoma virus in E. coli by Dr. Martin and the collaborative studies of tumorigenesis by polyoma DNA and restriction fragments thereof have led to important observations on the roles of various subregions of the early portion of the viral genome in polyoma tumorigenesis in hamsters. Also, cloning of C-type viral genomes in E. coli, is to be carried out in the near future by a collaborative group including NCI scientists and Drs. Martin and Rowe. This will be a major development in the C-type virus field and will permit rapid exploitation of many biochemical and genetic questions with an unheard of degree of precision and rapidity.

Two significant awards were made to LVD scientists. Dr. Rowe was named a co-recipient of the Paul-Ehrlich und Ludwig-Darmstaedter Prize for 1979, and Dr. Hartley was awarded the PHS Superior Service Award.

Some of the highlights of the scientific accomplishments of the past year follow.

New MCF-specific cell surface antigens detected. Antiserum prepared against the AKR247 isolate of MCF virus, tested by cell surface immunofluorescence absorption, detects antigenic specificities for ecotropic, xenotropic and MCF viruses. The MCF specificity has been shown to consist of 3 subtype-specific reactivities. Preliminary tests suggest that this antiserum can also be used to detect subtype differences between xenotropic MuLV strains. This type of serum may be of much use in defining leukemia-specific antigens in the mouse. (Cloyd, Hartley)

Only certain MCF MuLV isolates are leukemogenic. In tests of a number of cloned MCF isolates from various sources, only those isolates derived from inbred AKR mice have been active in acceleration of thymic lymphoma in AKR mice. Preliminary testing indicates that in contrast to the viruses from AKR, the non-leukemogenic isolates do not establish efficient infection in inoculated animals. (Cloyd, Hartley, Rowe)

Recombinant MuLV's recovered from mouse strains of both Fv-1^n and Fv-1^b genotype. Viruses with the characteristics of the class of dual tropic viral envelope gene recombinant viruses (MCF viruses), first detected in leukemic and preleukemic AKR mice, have now been isolated from 8 other high leukemia strains of mice. These include C_3H/Fg, NFS congenic for the ecotropic virus-inducing loci Akv-1, Akv-2, Fgv-1, and two loci from C58 (all Fv-1^n strains), and B10.A and BxH-2 (both Fv-1^b). Also, an MCF isolate has been recovered from a plasmacytoma induced in a BALB/c mouse, an Fv-1^b strain which develops spontaneous neoplasms only late in life. The mouse cell host range (N- or B-tropic) of each isolate is that for which the mouse strain of origin is permissive. (Hartley, Rowe)

Reinsertion of ecotropic MuLV into new chromosomal sites detected. In the course of inbreeding NFS mice congenic for a single ecotropic virus-inducing gene from AKR, reinsertion of the viral gene into 1 to 3 new chromosomal sites has been found. The reinsertion mouse strains will be used in studying whether viral integration occurs at random or preferred sites and the possible control by site of insertion of virus expression and development of disease. (Rowe)

New chromosomal locations identified for ecotropic and xenotropic MuLV's. Two ecotropic virus loci (Akv-1 and Fgv-1) were previously mapped at non-allelic sites on chromosome 7; locations for 3 additional ecotropic loci have now been identified, on chromosomes 5, 8, and 11. Loci controlling induction of xenotropic virus in 4 inbred strains have been mapped. All occur on chromosome 1 and appear to be allelic. (Kozak, Rowe)

JC virus replication in human amnion and human embryonic kidney cells. JC virus (JCV) is unusually host-restricted; since its discovery 8 years ago, the only cell culture system for replication of this virus has been human fetal brain cells. After testing numerous types of cells, we have achieved viral growth in human amnion through 7 passages. The virus has also been adapted to growth in human embryonic kidney cells. In both types of cells, the yields are not as high as in fetal brain; this appears to be due to a high proportion of defective interfering particles which are present in the stocks of JCV. (Takemoto, Miyamura)

Persistent BK virus infection in transformed fetal brain cells. A small fraction of human fetal brain cells which survived lytic infection with BK virus was recovered and propagated as permanent cell lines. These cells, which have been in culture for approximately 4 years, are persistently infected with BKV. They have many of the phenotypic properties of transformed cells such as growth in agar and low serum concentrations, indefinite growth, and tumor production in nude mice. Cloning experiments in antiserum-medium revealed that of 20 clones studied, all released virus after removal of antibody. After further regorous antiserum treatment, 3 out of 7 clones still released virus spontaneously after removal of antibody. Analysis of total cellular DNA of several cloned lines by the Southern blotting procedure showed that none of the clones contained integrated BKV DNA; instead, all lines carried free, episomal DNA in the form of circular (Form I) DNA. The cells are also unique in that they are T-antigen negative. This finding explains how the virus persists in culture, and also represents a novel, heretofore unrecognized type of cellular transformation by papovaviruses. (Takemoto, Miyamura)

Common papovavirus antigens. We have confirmed the observation of Shah, et al. that all members of the polyomavirus genus share a common antigenic determinant which is part of the major viral polypeptide, VP-1. In an extension of these observations we are developing more quantitative assays to determine the degree of relatedness of the different members of the group. These tests include immunodifffusion and radioimmunoprecipitation. Ultimately these data will be correlated with the findings on common DNA sequences shared by members of the group. (Takemoto, Bond)

Correlation of susceptibility of chicken cells to Entamoeba histolytica trophozoites in ovo and in vitro. We have shown previously that there are marked differences in the ability of eggs from different chickens or flocks to support the growth of E. histolytica. It has now been found that tissue culture cell lines from susceptible and resistant eggs demonstrate comparable susceptibility or resistance to the amoeba, even after ten passages in tissue culture. These cell lines offer a unique model system for the study at a cellular level of a genetically determined susceptiblity to E. histolytica. (Jaouni)

Further evaluation of an interferon inducer in primates. Preclinical and clinical studies with the interferon inducer, polyICLC, were continued last year. The drug continues to show promise of being of value in the treatment of human disease. While application of the drug to human viral disease awaits further long term toxicological and pharmacological studies, trails in non-human primates have been successful in a variety of serious viral infections. In particular, dramatic results have been obtained in post exposure prophylaxis of rabies in monkeys. In cancer patients, high levels of serum interferon have been induced with doses of drug that produce an acceptable degree of side effects. Dramatic improvement occurred during therapy of one patient with chronic relapsing polyneuropathy; further patients with this disease are now being treated. The drug acts as an immune adjuvant for certain weak vaccines, at levels of drug that are below those that have shown any undesirable effects. (Levy)

Serum neutralizable "toxin" of Entamoeba histolytica. Employing a rapid in vitro assay we have confirmed and extended the observation of Lushbaugh et al. of the presence of a serum neutralizable factor in E. histolytica cultures which produces rounding of tissue cultured cells. A serum component in adult and fetal bovine serum and in human serum neutralizes this toxin. We are studying the role of the toxic factor and of the neutralizing component in disease production in vivo. (Mattern; Keister, LPD; Natovitz)

Selection of stable virulent subpopulations of E. histolytica. A nearly avirulent strain of axenically cultured amebae has been increased 1000-fold in virulence by alternate passages in the liver of the newborn hamster and in axenic culture medium, with the selection of liver lesions produced at limiting dilutions. Such isolates have remained highly virulent for many months. (Mattern; Keister, LPD)

Beaded amebal virus DNA. The 14 bead amebal virus was purified in CsCl density gradients and found to be a DNA virus by virtue of its incorporation of labelled thymidine and exclusion of labelled uridine (Mattern; Keister, LPD). The nature of its DNA is being further explored in collaboration with Dr. Defilippes (LBV).

Virulence assays for Trichomonas vaginalis. A neonatal animal test and a tissue culture assay for virulence of I vaginalis both appear highly promising and are being developed for a new program on this parasite (Mattern, Natovitz).

Isolation of AAV-Type 1 from commercially prepared human embryonic kidney (HEK) cells. AAV-1 was repeatedly found as contaminant of HEK cells during a period when such cells were prepared in close proximity to Rhesus monkey kidney cultures. No further isolations were made after HEK cultures were prepared on different days and in separate cubicles from the Rhesus monkey cultures. These results point out that human cell cultures can be readily cross contaminated with AAV-1 from simian tissues and that AAV-1 is of simian origin. (Hoggan, Sears)

AAV-2 genome integrates at a specific site on host cell genome. We have previously shown that Detroit-6 AAV-2 carrier cell clones contain 3-5 genome equivalents of AAV-2 DNA per host cell genome. Current studies show that these multiple copies are integrated at a single site on the host cell DNA. Whether

the integration site is the same for all AAV-2 carrier clones as well as for other AAV type carrier cells is currently under study. (Berns, Hoggan)

N-terminal amino acids of AAV-blocked with acetate group. While attempting sequence the amino acids at the N-terminal end of each AAV polypeptide it was discovered that each was blocked with an acetate group. Similar findings have been reported for adenovirus as well as certain phage polypeptides while they have rarely been encountered with non viral proteins. Significance of these findings are unknown but it is suggested they may have something to do with virus assembly and/or post assembly maturation. (Johnson, Hoggan)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00011-13 LVD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies of Small DNA Containing Viruses Belonging to the Family Parvoviridae.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI : M. D. Hoggan Staff Scientist, LVD, NIAID
Other: A. Roy
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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SUMMARY OF WORK (200 words or less - underline keywords)

Studies designed to delineate the mechanisms of parvovirus infection and persistence and their possible role in disease have continued using classical virological and immunological as well as modern biochemical technologies. Using our previously developed AAV carrier systems we have determined that multiple copies of the AAV genome integrate into the host cell genome. Further studies using restriction enzyme analysis and heteroduplex mapping should provide insight into the mechanisms of integration and persistence of the AAV genome as well as the processes of excision and productive infection in the presence of helper virus. Investigation will continue of both the structural and virus coded non-virion proteins of the AAV as well as comparative polypeptide and DNA studies of the various plaque and virulence variants of Rat virus, Lu III, MVM, and PVR⁻ we have isolated. These findings, in concert with previous studies of the immunologic specificities of various parvovirus proteins, may well allow the complete mapping of a number of different parvovirus genomes.

Project Description

Objectives: To employ recently developed biochemical and immunological technology as well as traditional physical-chemical, immunological and epidemiological procedures to the study of viruses belonging to the Family Parvoviridae in order to gain a better understanding of their replication, persistence, natural history and evolution as well as their possible role in disease.

Major Findings

Evidence that the Rhesus monkey may be the primary host for AAV-type 1: We previously reported that approximately 0.5 to 1.0% of all human embryo kidneys contain AAV type 2 in an occult form and AAV types 2 and 3 can be isolated from children. On the other hand AAV types 1 and 4 are usually found in association with adenoviruses grown in various monkey kidney cultures even though AAV-type 1 can be readily transferred to adenoviruses grown in human cells. After many years of never having been able to isolate AAV-1 from HEK cultures we recently found 7 out of 10 lots of HEK cells from one commercial house to be contaminated with this virus. We were unable to isolate AAV-1 from any of the materials used to prepare the cultures, however, after a careful review of the procedures used in their preparation it was found that during the period when the isolates were made, the technicians had prepared both Rhesus and human kidney cultures in the same cubicle. Once this practice was stopped no subsequent "contamination" of HEK cultures with AAV-1 occurred. These findings support the suggestion by us and others, that AAV-1 is probably of monkey origin.

Further evidence that the AAV-2 genome is integrated into the host genome of the Detroit-6 carrier cell clones: In collaborative studies carried out with Berns et al. we have previously reported that our occult AAV-2 carrier culture clones of Detroit-6 cells contain 3-5 AAV-2 genome equivalents per cell and that this DNA is probably integrated. Recent studies using the blotting technique for in situ hybridization have shown that not only is the AAV-2 DNA integrated into host cell DNA but multiple copies of the AAV-2 DNA are integrated at a single specific site on the host genome. These results are in marked contrast to the results obtained for SV40 integrated into host cell DNA.

Studies of parvovirus proteins: During studies designed to elucidate the N-terminal amino acid on each of the three recognized AAV structural polypeptides, we have found that they are blocked with an acetate group and can not be sequenced using the Dansyl chloride technique. We have further found that when AAV is grown in the presence of C^{14} labeled acetate a 120,000 MW polypeptide is found in association with heavy (>1.46 g/cm^3 in CsCl) particles. A precursor virus polypeptide of similar size has been reported by Johnson in infected cell lysates.

Further studies of a large plaque rat virus variant (RVLP) containing MVM antigen(s): We previously reported the isolation and characterization of a variant of rat virus which not only produces larger plaques (>5 mm vs <2 mm

for wild type plaques) but contains a cross reacting MVM antigen(s) and has a reduced virulence for newborn hamsters. We noted previously that plaque pools of small plaque virus breed true while pools of large plaque virus always contain both large and small plaques. We have recently been able to produce pools of large plaque virus containing very few small plaque particles by treatment with limiting dilutions of antiserum made against purified complete small plaque particles. Studies on the characterization of the DNA from the two populations using restriction enzyme analyses of double stranded DNA made in vitro are in the preparative stages. Of interest is the finding that hyperimmune antiserum prepared against empty MVM particles does not contain cross reacting antibody to RVLP even though extremely high levels of homologous MVM antibody are produced.

Isolation and characterization of AAV-1 and AAV-2 "clones": We previously reported the isolation of "statistical clones" of AAV-2 and noted that they were indistinguishable from AAV-2 isolated from AAV-2 carrier cells in terms of their biological characteristics as well as their DNA. We have also found that low passages of such clones contain a high percentage of infectious virus while recent studies by others have found that serial passage of AAV in cell culture results in the production of a large percentage of defective (DI) particles. The need for low passage AAV-type 1 and 3 stock "clones" in addition to our AAV-2 stock clones has thus become apparent and we are now preparing them for future work.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AI 00012-16 LVD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of Mouse Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Dr. Sisir K. Chattopadhyay, DCT, NCI
Dr. Akinori Ishimoto, LVD, NIAID (Visiting Associate) (Until June 1978)
Dr. Eli Canaani, LVD, NIAID (Until February 1978)
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COOPERATING UNITS (if any) L. Old, E. Stockert, Sloan Kettering; H. Morse, LMI, NIAID; T. Chused, OMS, NIDR; M. Collins, S. Hourihan, Microbiol. Assoc., Inc.; P. Pithal, S. Staal, Johns Hopkins Univ.; M. Potter, LCBGY, NCI; N. Hopkins, MIT

LAB/BRANCH
Laboratory of Viral Diseases

SECTION
Viral Oncology Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
13.5	5.7	7.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The natural biology of the complex group of murine C-type RNA viruses is being studied using virological, genetic, and biochemical approaches. Transmission by genetic means has been delineated for a number of spontaneously produced or chemically inducible murine leukemia viruses (MuLV); these include xenotropic and ecotropic MuLV from both high and low virus expression mouse strains. In addition, the chromosomal location of several of these virus genomes has been identified. In studies of the relation of this virus group to neoplastic disease in mice, naturally occurring recombinant viruses containing determinants of both ecotropic and xenotropic MuLV have been found. These viruses are of particular interest because of their association with the preleukemic or leukemic state, the ability of certain isolates to induce or accelerate leukemia in mice, and the opportunity they provide for analyzing virus genome structure and segment function.

This project is a long-term study of the natural biology of C-type viruses of mice, with particular emphasis on their transmission as chromosomal genes and on the genetic factors that affect their expression in vivo. The major approaches to these problems are the breeding of hybrid and congenic mice and the characterization of their endogenous virus expression by various tissue culture assays.

Chromosomal mapping of endogenous genomes. A major objective is to map the chromosomal location of the various endogenous genomes. By means of mendelian and somatic cell hybrid genetic approaches, we have now mapped ecotropic virus-inducing loci in five inbred mouse strains, and xenotropic virus-inducing loci in four. The ecotropic genomes are not at allelic sites in the various strains, but all four mapped xenotropic loci are on chromosome 1, probably at the same site. Whether this region of chromosome 1 contains the structural genes of the xenotropic viruses or is regulatory is not known.

Congenic mouse strains. An important component of the program is the development of congenic mouse strains. These are chiefly strains in which a virus-inducing locus is being bred into a normally "virus-negative" genetic background, but we are also beginning to make strains that are congenic for the absence of virus. In particular, we will make lines of BALB/c which are negative for ecotropic and/or xenotropic virus expression. These strains will be of great importance in analysing the role of endogenous virus expression in various types of carcinogen-induced and spontaneous tumorigenesis.

New chromosomal insertions. In the course of inbreeding the partially congenic lines of mice, we have twice observed the reinsertion of ecotropic virus genomes from the Akv-1 locus into new chromosomal sites. This was evidenced by the finding that backcrosses of the homozygous Akv-1 congenic mice to a "virus-negative" strain did not give 50% positivity, as expected for a single locus, but gave 75 to 90% positives, indicating that there are now two to four unlinked loci in the strains that initially had but one. Breeding of these loci into separate families has confirmed that some are not at the Akv-1 site on chromosome 7.

These new reinsertion lines offer unique experimental material for studying whether viruses insert into preferred sites, into preexisting viral sequences, or randomly, and whether the sites of insertion play a role in virus expression and its production of disease.

Origin of B-tropic virus. Several recent studies from other laboratories, based largely on virus strains from LVD, have indicated that B-tropic MuLV arises from N-tropic virus by genetic recombination in the gene for the major core protein, p30. In studies of the Sloan-Kettering AKR-Fv-1^b mouse, which is a high virus AKR mouse into which the Fv-1^b allele has been bred, we have found that these mice regularly generate B-tropic virus at 6 to 8 months. Through study of these mice we will be able to determine if the thymus plays a crucial role in this type of recombination as it does with MCF virus formation.

Characteristics of mink cell focus-inducing (MCF) murine leukemia viruses (MuLV). Viruses of this class have in common a dual host range (i.e., mouse and certain heterologous species cells), antigenic relationship (but not identity) to both ecotropic and xenotropic classes of MuLV, and an association with murine leukemogenesis. All isolates tested to date appear to be recombinant viruses, arising uniquely in the animal of origin, from endogenous ecotropic virus and some other endogenous MuLV sequences presumably related to xenotropic MuLV's but not yet identified. The biological and serological properties of the viruses, tryptic digest peptide analysis of viral envelope and internal proteins (done in collaboration with J. Elder and R. Lerner), and oligo-nucleotide mapping of T1 RNase digests of viral RNA (done in collaboration with N. Hopkins), indicate that the recombination involves viral gene segments coding for envelope properties, that the internal virion proteins are derived from the ecotropic partner, and that each isolate may be unique.

Nucleic acid homology studies indicate that approximately 90% of the MCF virus genome is represented in cDNA probes specific for the AKR ecotropic virus genome. Preliminary tests suggest that it may be possible by hybridization saturation comparisons to identify the putative xenotropic parental virus or viral sequences involved in generation of a particular recombinant virus.

MCF virus isolations. A number of isolates have now been obtained from mice of the high N-tropic ecotropic virus, high leukemia phenotype, including AKR, C3H/Fg, and NFS congenic for the ecotropic loci Akv-1 (two independent congenic lines), Akv-2, and ecotropic loci from C3H/Fg and C58 (two lines). These isolates, all from Fv-1^n mouse strains, are N-tropic in their mouse cell host range. In contrast, B-tropic MCF isolates have been obtained from two Fv-1^b mouse strains (B10.A and BxH-2), both of which express high levels of B-tropic ecotropic MuLV early in life and have a high frequency of spontaneous leukemia. In addition, an isolate of B-tropic MCF-type virus has been recovered from a BALB/c mineral oil-induced plasmacytoma.

Leukemogenicity of naturally occurring MCF and ecotropic MuLV. Tests of these viruses for ability to accelerate the development of thymic lymphomas in AKR mice have been carried out by intrathymic inoculation of suckling or weanling mice. The 4 MCF isolates from AKR mice tested to date have been highly active in this assay, in that all inoculated mice developed leukemia within 2 to 6 months of age, whereas lymphomas in control mice developed only at age 7 1/2 to 11 months or older. In marked contrast, none of the groups of AKR mice similarly inoculated with 8 different MCF isolates from NFS mice congenic for the AKR, C58, or C3H/Fg ecotropic MuLV loci showed a significant shortening of the latent period, nor did mice inoculated with AKR-derived ecotropic viruses. Similarly, AKR-derived MCF's accelerated lymphoma in high-virus Akv-1 and Akv-2 congenic mice, but viruses derived from these strains did not. In limited testing in other strains of mice (C3H/Bi, DBA/2, C57Br and NFS - all low or negative ecotropic virus Fv-1^n strains), only the AKR-247 MCF isolate has been leukemogenic and that only in C3H/Bi.

Testing for virus in the preleukemic period indicates that AKR mice inoculated with the leukemia-accelerating isolates regularly have a high percentage of MCF

virus-producing cells in thymus, while mice inoculated with other MCF isolates are much less consistently positive and at a lower level.

The ability of different MCF isolates to establish efficient in vivo infection is presumably a major variable in determining leukemogenicity. More extensive comparisons of additional isolates are planned to identify correlates of a particular biological activity and to identify host factors controlling distribution and level of virus.

Although the association of MCF viruses with neoplastic disease is of intrinsic interest, these recombinant viruses also provide a means for studying the organization of the MuLV genome, for identification of the biological function of various gene segments, and for elucidation of the mechanism of recombination.

MCF-specific cell surface antigens. Using cell surface immunofluorescence absorption assays and rabbit antiserum prepared against the AKR247 MCF isolate, we have detected cell surface antigenic reactivities specific for ecotropic, xenotropic and MCF virus infection. The MCF specificity has been shown to comprise three subtype-specific reactivities: 1) MCF group reactivity, demonstrable on cells infected with all MCF isolates tested, including those for representative naturally occurring viruses as well as MCF-type isolates of Moloney and Friend virus origin; 2) reactivity specific for the naturally occurring isolates and 3) a subtype specificity restricted to only certain MCF isolates. Although no correlations between these antigenic markers and source or biological properties of the isolates have been found to date, the technique provides another means for comparison and identification of MCF isolates and may allow detection of expression of MCF-specific proteins in studies of the pathogenesis of murine leukemia.

Virology of BALB/c plasmacytomas. A collaborative study with M. Potter is in progress to define the spectrum and level of MuLV activity in BALB/c mineral oil induced plasmacytomas and pre-tumor peritoneal exudate cells. As noted above, a B-tropic MCF virus has been isolated from one tumor; its possible relation to the etiology of plasma cell tumors is being investigated.

Genetics of MuLV in NZB mice. Studies in collaboration with T. Chused and H. Morse are in progress to establish strains of NFS mice congenic for two independent loci controlling xenotropic virus production in NZB mice, the only mouse strain known to produce a high level of this class of MuLV, as well as a probable third locus involved in production of a xenotropic virus associated cell surface antigen (XenCSA) which can be detected in the absence of infectious virus. These congenic mice should prove useful in studies of NZB autoimmune disease, in lymphoid cell differentiation studies, and in studies of factors controlling expression of xenotropic virus.

Sub-group characterization of xenotropic viruses. A number of studies from this and other laboratories indicate that xenotropic viruses from various sources although closely related are not identical. Subtype differences have been suggested by analyses of nucleic acid hybridzation, virion proteins, and differences in host range pattern. In addition, preliminary cell surface immunofluorescence

absorption studies using anti-MCF antiserum indicates a possible additional means of detecting xenotropic virus diversity. Comparison of xenotropic viruses from various sources by several of these techniques may provide a meaningful classification system.

Publications

Chattopadhyay, S.K., Levine, A.S., and Rowe, W.P.: Presence of naturally occurring murine leukemia viral sequences in mouse chromosomes and their correlation with murine leukemia. 1977. Proceedings of International Symposium on the Molecular Basis of Host-Virus Interaction. Benares, India, October 17-20, 1976.

Chattopadhyay, S.K., Lander, M.R., Kramer, B.S., Hartley, J.W., Levine, A.S., and Rowe, W.P.: The use of nucleic acid hybridization to demonstrate relatedness between the naturally occurring and laboratory strains of murine C-type viruses. Advances in Comparative Leukemia Research 1977, eds. Bentvelzen et al., 1978 Elsevier/North-Holland Biomedical Press, pgs. 448-451.

Chattopadhyay, S.K., Levine, A.S., and Rowe, W.P.: Biochemical detection of murine leukemia virus genomes in mouse chromosomes. The Nucleus 20: 182-193, 1977.

Chattopadhyay, S.K., Hartley, J.W., Lander, M.R., Kramer, B.S., and Rowe, W.P.: Biochemical characterization of the amphotropic group of murine leukemia viruses. J. Virology 26: 29-39, 1978.

Elder, J.H., Gautsch, J.W., Jensen, F.C., Lerner, R.A., Hartley, J.W., and Rowe, W.P.: Biochemical evidence that MCF murine leukemia viruses are envelope (env) gene recombinants. Proc. Natl. Acad. Sci. 74: 4676-4680, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00015-12 LVD

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Structure, Properties, and Replication of Viruses of Pathogenic Protozoa and Mammals.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	W. A. Daniel	Biologist	LVD, NIAID
	D. B. Keister	Biologist	LPD, NIAID

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LAB/BRANCH

Laboratory of Viral Diseases

SECTION

Cellular Virology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

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3-3/12	2-3/12	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to evaluate a variety of factors which may influence the virulence of the pathogenic protozoa Entamoeba histolytica and Trichomonas vaginalis:

An ongoing study on E. histolytica (in collaboration with LPD) is evaluating the roles of amebal viruses, selection pressures exerted by axenic cultivation and animal passage, and a serum-neutralizable amebal toxin in the variation in virulence among different amebal strains.

A similar program of study Trichomonas vaginalis (in collaboration with Dr. B.M. Honigberg, Univ. Mass, Amherst) is in its early phases. We are developing virulence assays both in the newborn mouse and in tissue cultures. The program will be expanded to search different isolates for viruses, plasmids, other extrachromosomal elements or possible interaction with human wart viruses.

Further characterization of amebal viruses has revealed that the beaded virus BV_{200} consists of 14 beads as the monomeric unit, incorporates labelled thymidine and excludes uridine and thus represents another DNA amebal virus.

Project Description

The major emphasis of this program in the past year has been to identify factors responsible for variation of different strains of Entamoeba histolytica, including viruses of E. histolytica.

In the past 6 months we have employed the in vitro tissue culture model to confirm and extend studies on a serum-neutralizable toxin of E. histolytica, originally described by Lushbaugh et al., University of South Carolina, with whom we are in close contact in order to avoid duplication of effort. The quantity of toxin per ameba is proportional to the virulence of the amebal strain. The toxin produces a rapid rounding of tissue cultured cells (15 min. - 1 hr.), yet, the cells are not killed in this time since they will survive more than 20 hr. of exposure, if washed and refed fresh tissue tissue culture medium. We intend to identify the serum fraction(s) responsible for "neutralizing" the toxin. To date we have localized the neutralizing factor in the 40 and 50% ammonium sulfate fractions of bovine serum. We intend to evaluate the role of toxin in virulence of amebae, and ascertain if the toxin neutralizing factor offers protection against virulent amebal strains. This has possible therapeutic implications (Mattern, LVD; Keister, LPD; Natovitz, LVD; Daniel, LVD).

A nearly avirulent strain of E. histolytica, Rahman, has been dramatically increased by 3 logs in virulence by alternate passages in the liver of the newborn hamster and in axenic culture medium with the selection of those liver lesions produced at limiting dilution. Futher studies are planned on possible generality of this phenomenon. This has practical importance in permitting the prolonged axenic cultivation of virulent strains by occasional liver passage. Clones of virulent amebae will be followed to ascertain if they maintain or lose virulence, since uncloned populations usually lose virulence with prolonged axenic cultivation. It is not known whether the virulence loss represents changes in the amebae or selection of perhaps a faster growing avirulent subpopulation from an uncloned population (Mattern, LVD; Keister, LPD; Natovitz, LVD).

Viruses of Entamoeba histolytica.

We have shown that the purified beaded virus of E. histolytica consists of double stranded DNA by its acridine orange staining and its incorporation of ^{3}H-thymidine and exclusion of ^{3}H-uridine. We intend to purify moderate quantities of virus for structure analysis and DNA analysis. If the virus can be crystallized or at least oriented in gels (as was done with

tobacco mosaic virus) it can be examined by x-ray crystallography. The beaded viruses of the amebal strains Rahman and 200 both consist of 14 beads as the monomeric unit. Antisera will be prepared to both to evaluate host range differences being reflected in immunological differences. We Will also attempt to purify the flexible filamentous amebal virus as well as a new and unreported rigid filamentous virus-like particle which appears to replicate in the nucleus and lyse amebae.

Dr. Thomas F. McCaul, a visiting fellow at the RML, will probably spend his last year in my lab trying to identify and isolate the unusual bar-like structure of E. histolytica which seems to have phage-like properties (Mattern, LVD; Leister, LPD; Diamond, LPD; Natovitz, LVD).

Trichomonas vaginalis.

Preliminary studies indicate that newborn mice inoculated subcutaneously are very much more susceptible to T. vaginalis than are adult mice and that a tissue culture assay of the virulence of the parasite is possible.

We intend to examine trichomonads for viruses or plasmids and to investigate T. vaginalis for transfer of virulence to an avirulent strain by the DNA and RNA of the former, as observed by Honigberg et al. in T. gallinae (Mattern, LVD; Natovitz, LVD; Daniel, LVD; Honigberg, Un. Mass.).

Human wart viruses (HWV).

We have initiated attempts to culture human wart cells, isolate the viruses of warts, and develop animal models for the study of HWV. Possible interactions of Trichomonas vaginalis and the HWV will be investigated (Mattern, LVD; Natovitz, LVD; Daniel, LVD; Normura, NCI; Abramson, NNMC).

Clinical study on mechanism of taste transduction.

It is hoped that this study can be concluded and phased out within the next 1-1/2 years. The study is in progress and involves a minimal amount of time, 2 - 3 hrs/week (Mattern, LVD; Wolf, NIDR, Weiffenbach, NIDR; Kapp, Clinical Center anesthesiology service).

Viral capsomeric images.

In view of the great difficulty in obtaining single sided images of viruses with negative staining we are attempting to devise tehniques for obsuring one surface of the virus from the

negative stain so that we can observe only one surface. The significance is that in the triangulation number T=7 group of viruses, including all papovaviruses, single sided inages are needed to determine whether the virus has 72 capsomeres and whether they are dextro or levo T=7 viruses (Daniel, LVD; Mattern, LVD).

Publications

Mattern, C.F.T., Keister, D.B., Caspar, P.A. Experimental Amebiasis III. A rapid in vitro assay for virulence of Entamoeba histolytica. Am. J. Trop. Med. Hyg. in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00018-12 LVD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Biological and Biochemical Characterization of Human Papovaviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Tatsuo Miyamura	LVD, NIAID

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3	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The primary research goals of this laboratory have been the biological and biochemical characterization of the new human papovaviruses, BKV and JCV. Although much has been learned about BKV, little is understood about JCV due primarily to difficulties in propagating the virus in readily available cells. Efforts have therefore been directed toward obtaining a suitable system for JCV growth, and we have achieved some success in propagating the virus in human amnion cells. Further efforts are being made to increase the yields.

Since both human papovaviruses cause persistent infection in their hosts, we have attempted to study BKV persistence in cell culture. A system of persistent infection in human fetal brain cells was successfully established and the mechanisms of viral persistence were studied. The persistently infected cells are phenotypically transformed, but possess properties which are unique; they lack T-antigen and viral DNA is present in all cells in the form of episomal DNA. These cells thus provide new insights into mechanisms of viral persistence and transformation.

PHS-6040

Project Description

Replication of JC virus in human amnion cells. JCV has not been grown in cells other than human fetal brain cells. This narrow host restiction has severely limited studies on this important virus. While others (Padgett, et al) have reported that JCV does not grow in any of a large number of human, monkey, and rodent cells tested, we have recently been able to propagate this virus successfully through 6 passages in primary human amnion and 3 passages in human embryonic kidney cells. By restriction nuclease analysis of viral DNA, as well as by serologic methods, the virus grown in amnion cells has been shown to be the same as the prototype virus. Although the yields are low, they now allow us to do further biological and biochemical investigations on this virus.

Persistent BK virus infection in tissue culture. Both human papovaviruses, BK and JC, cause persistent infection in humans. How these viruses persist is not understood. We have therefore attempted to set up experimental models in tissue culture, based on previous information with SV40 and polyoma. Infection of permissive or semi-permissive cells with these viruses usually results in persistent infection in the surviving cell population accompanied by cell transformation. In the case of BKV in highly permissive fetal brain cells, the vast majority of the cells are killed by the virus, but rare survivors can be recovered. These surviving cells can be established as permanent transformed lines, and virus persists indefinitely in a small percentage of the cells, depending on the state of the culture. Cloning experiments in antiserum-medium have shown that the virus does not persist in a simple carrier state. Of 20 clones studied, all began producing virus when antibody was removed. After rigorous antibody treatment, 3 out of 7 clones still released virus spontaneously, while the other 4 have remained virus free.

Analysis of the DNA from 7 clonal lines using the Southern procedure have revealed that all contain episomal DNA and no integrated sequences. The DNA is in the form of free, twisted circular Form I molecules. This finding of episomal DNA in papovavirus transformed cells is unique and somewhat resembles EBV and H. saimiri lymphoblastoid lines. However, a major differences is the lack of T-antigen in the BKV transformed cells, while the herpes lines produce EB nuclear antigen (EBNA).

Common papovavirus antigens. All papovaviruses of the polyomavirus genus share a common antigenic determinant residing in the VP-1 poplpeptide. This can be readily demonstrated with the use of antiserum against disrupted virions. We have also shown that antibody against human papilloma virus does not react with this common antigenic determinant. Previous tests have utilized the immunofluorescence technique to demonstrate cross-reactivity; this test does not provide highly quantitative information. Therefore, other methods involving immunodiffusion, radioimmunoprecipitation, etc., are being investigated to determine degrees of reactivity of the capsid proteins of the various members of the group.

This study is being carried out in conjunction with other studies in collaboration with P. Howley to determine the localization and extent of common sequences among the polyomaviruses (BK, JC, KV, SV40, and polyoma). Evidence obtained thus far indicates the common sequences to be in a small region of the DNA coding for VP-1.

Significance to Biomedical Research

The human papovaviruses, BK and JC, infect children at an early age and persist thereafter throughout life. Although BK has not been shown to cause disease thus far, the JC virus has been associated with over 30 cases of PML and appear to be the causative agent for this disease. The papovaviruses thus represent a class of persistent viruses whose role in chronic disease need to be elucidated. There oncogenicity in vitro and in vivo also provide investigators with important models for studies on mechanisms of viral oncogenesis.

Proposed course

We will continue efforts to propagate JC virus in cells other than those from fetal brains, and the immediate objective is to obtain more efficient replication in human amnion cells. The virus yields are low at the present time. Once higher yields are achieved, then detailed biological and biochemical studies are planned. The system of persistent BKV infection in T-antigen negative, transformed human fetal brain cells will continue to be investigated. The questions still to be answered are: (1) site and number of episomal DNA in the cells; (2) whether there is transcription off the free, episomal DNA; and (3) mechanisms of spontaneous induction in virus-releasing cells.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00019-04 LVD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on the treatment of disease with the interferon system.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any) Dr. G. Baer, CDC; Maj. E. Stephen, Capt. L. Sammon, Maj. D. Harrington, Capt. E. Houston, Ft. Detrick; Dr. M. Lerner, Wayne State U. Sch. of Med; Dr. A. Levine, NCI; Dr. R. Guerra, U. of Sienna Med. Sch., Sienna, Italy; Dr. C. Anfinsen, NIH, Dr. W.K. Engel, NINCDS.

LAB/BRANCH
Laboratory of Viral Diseases

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	4	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is aimed at applying the interferon system to the treatment of human disease. Previous reports outlined that a primate-effective interferon inducer has been developed, and has been successful in treating a variety of diseases in monkeys. A phase I study indicate that 350 µg/kg body weight is the highest level of drug that can be administered to cancer patients with acceptable levels of toxicity. This level of drug yields mean peak serum interferon levels of 2000 µ/ml, about 6-10x that is obtainable with exogenous interferon Effective post exposure prophylaxis of monkeys injected with LD_{100} of street rabies can be accomplished with 1 dose of the drug plus one dose of vaccine; a more effective regime than vaccine and anti-rabies serum. The drug is acting as an immune adjuvant in very small quantities that are well below the levels that elicit any toxicity at all.

Project Description

The work of the past year may be considered in 3 parts, all dealing with the drug, polyICLC.

Drug preparation. We have been fortunate in having been able to avoid contamination, and to prepare a pharmaceutical grade material. However, if the envisioned large scale trials develop, it may prove difficult for us to manufacture enough material.

During the earlier stages of this work, we ran into some problems because one manufacturer mislabelled the molecular weight of poly lysine that they supplied. This led to our having made some inactive preparations. Poly lysine of at least 13000 Daltons is needed for a satisfactory preparation and 17000 to 27000 is best. We have standardized on this now.

After discussion with Dr. Felsenfeld, the person who first made polyI.polyC, we have come up with a tentative structural formula for polyICLC, in which the purpose of the CMC has been to stretch out the poly lysine into a linear form, so that when polyI.polyC enters the complex, branching, with associated insolubility does not occur.

Animal Studies

With one exception the drug has had beneficial effect in every monkey model that has been examined. This includes Simian hemorrhagic fever, Japanese B encephalitis, rabies, pichinde virus, kerato-conjunctivitis, Venezuelan equine encephalitis, chronic hepatitis B (chimpanzees), Russian Spring Summer encephalitis, vaccinial keratitis and systemic vaccinia infection. It did not work in Machupo infection, and actually may have made the disease worse. This last experiment should be repeated without newer preparation, because the negative experiment was done at a time when some of our preparations were ineffective.

In monkeys as well as mice, the drug is an effective immune adjuvant when given with at least certain weak antigens, specifically swine flu vaccine, Venezuelan equine encephalitis virus vaccine and H. influenzae polyribose phosphate vaccine. In these cases humoral antibodies are increased up to 100 fold. However, when given with a good antigen like albumin, there was actually an inhibition of antibody production. The immune enhancing effects were seen at levels of drug which are below those needed to produce interferon or a pyrogenic response.

Studies in man

In collaboration with Dr. Levine of NCI, we have completed a phase I study in terminal cancer patients, to obtain a dose-response curve as well as to determine what is the highest tolerated levels of polyICLC. Doses of drug up to 800 µg/kg body weight were given to at least 4 separate patients at each level (15 injections per course). At levels of 800 µg/kg, peak serum titers

of 15000 units/ml of serum were seen, probably the highest man has ever made, but these levels of drug (or interferon?) were associated with an unacceptable amount of toxicity, including hypotension, myalgia and arthralgia. However, at 400 µg/kg body weight, the degree of side effects were considered acceptable. Mean peak serum interferon levels of 2000 units/ml serum were obtained. While the study was not designed to test therapeutic effect - all patients were very far advanced - there was one case of acute lymphocytic leukemia who was placed into complete remission by the drug, and another went into partial remission.

With Dr. Engel of the Neurological Disease Institute, we are examining the effects of the drug in a number of neuromuscular disorders. With one patient there has been a dramatic improvement. Confirmatory studies are under way with encouraging results so far. Levels of drug used have been 50-100 µg/kg body weight. The amount of serum interferon generated has been just slightly higher than those seen by Dr. Levine.

With Dr. Lerner we have done a small study of examining the reactions of people to polyICLC. In the study with Dr. Lerner, we find about the same level of interferon for a given dose of drug, and we still have ongoing a study on topical treatment of herpes genitalis.

The World Health Organization Expert Committee on Rabies has recommended that the drug be tried in rabies post exposure prophylaxis in humans.

Future plans

It is hoped to be able to do some studies in the therapy of viral diseases in man. Candidate diseases would include hepatitis, rabies, respiratory disease, chronic herpes kerato-conjunctivitis. For at least some of the studies it is felt that more information about the pharmacology and toxicologic properties of the drug needs to be obtained, both in animals and man. Animal toxicology of acute administration and short term chronic administration in monkeys and man has been done.

These studies were designed to give maximum information on chronic administration, but more remains to be done on really long term effects. We have prepared a first draft of an RFP which will deal with the animal studies. However, different species of animals differ in their reaction to the drug, and additional human studies are needed. Four groups of investigators are preparing protocols to be submitted for NIAID clearance for human studies in cancer patients. This should provide very valuable information for future virus work. The institutions are: Sloan Kettering Institute, M.D. Anderson Hospital, South West Testing group (a consortium of about 50 institutions) and the NCI. Interest has been heightened because 1) several groups have reported positive results in treating leukemia and lymphoma with interferon; 2) interferon is virtually not available; 3) polyICLC can induce large quantities of interferon with acceptable side effects.

At present more information is needed about the pharmacology and toxicology of the drug in man, including modulating effects on the immune system, and alterations of T and B lymphocytes.

Long term studies on improvement of the drug are beginning. Such a possibility has developed from experiments we have done with different methods of combining the components. It may be possible to prepare a poly lysine complex of polyI.polyC without carboxymethylcellulose. We have some evidence that this may indeed be so. However, a good deal of work is needed before we can so conclude, and Dr. Galasso and I feel that the testing of the standard compound should go on while the modified version is being developed.

We are also conducting work on the molecular mechanism of action of interferon, measuring changes in membrane proteins, and in protein synthesizing systems in interferon treated cells. At the present time there is nothing of significance to report.

Optimum conditions for growth of and interferon production by a human lymphoblastoid cell line, Namalva, have been studied. Adaptation to large-scale production is possible utilizing either Sendai virus or Newcastle disease virus (NDV). Priming of culture before induction is unnecessary. The interferon produced has properties similar to human leucocyte interferon. The production of lymphoblastoid interferon per cell is increased two to four fold after dilution with serum free medium of a saturation-density culture of Namalva cells induced with NDV. Maximum interferon yields were obtained 27 hours after addition of virus, using cultures diluted to $4x10^5$ to $9x10^5$ cells per ml. The presence of glutamine in the dilution medium was required for maximum interferon production. NDV appeared to inhibit the rates of RNA and protein synthesis more effectively in the diluted cultures.

Interferon produced on an 800-liter scale ($2.6x10^9$ units) was partially purified by anti-leukocyte interferon affinity chromatography, sulfopropyl Sephadex ion exchange chromatography, isoelectric focusing, and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Recovery of interferon after gel electrophoresis varied from 11 to 33% based on the original crude material, with about 35,000-fold purification. The gel electrophoresis resolved the antiviral activity into two components with apparent molecular weights of 18,000 and 22,000; treatment with glycosidases resulted in all the activity being associated with the lower molecular weight species. Interferon activity could be completely (85 to 113%) recovered from the gels by eluting it in a buffer containing SDS. The presence of SDS did not appear to affect the assay of interferon. The protein could also be stained with Coomassie blue, again with no loss in activity.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00165-02 LVD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biology and Immunology of Viruses Associated with Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The major goals are: 1) to determine the role of viruses of parasitic protozoa in pathologic conditions in humans, 2) definition of genetic factors contained in Entamoeba histolytica which determine host range and/or cell lysis, and 3) confirmation of genetic basis of variation in host range to E. histolytica in the absence of cell mediated immunity and and humoral antibody. The immediate goals are: 1) development of serologic tests and immunological definition of the protozoal viruses presently identified, 2) purification of the icosahedral virus(es) which have been associated with all E. histolytica strains studied, 3) identification of group and type specific antigens of the beaded viruses of E. histolytica, 4) determination of the occurrence and possible role of the viruses of E. histolytica in human population groups, 5) characterization of autolytic toxins associated with certain strains of pathogenic amoeba, 6) identification of the mechanism(s) responsible for susceptibility and resistance in avian tissue, 7) development of genetically stable E. histolytica sensitive and resistant chicken flocks and 8) definition of how the genetic constitution of a host may change the amoebae, and how trophozoites may be altered by multiple passages in resistant and sensitive hosts.

Project Description

Methods Employed: Tissue culture, axenic cultivation of parasitic protozoa, ultrafiltration, ultracentrifugation, electrophoresis, isoelectric focusing, chromotography, electron-microscopy, epidemiology, genetics, immunologic and serologic systems, including serum neutralization, agar-gel precipitation, gel-electrophoresis, fluorescent-antibody and ELISA.

Genetic control of susceptibility to E. histolytica in avian tissues: The initial attempts to establish axenically grown E. histolytica in embryonated hen's eggs were made in an effort to develop a bioassay system not requiring the use of mammals. It was hoped, also, that a method of cultivating amoebae axenically, not requiring the use of large amounts of bovine serum, could be developed for use in immunologic studies of E. histolytica and for the viruses associated with it. Preliminary studies, however, revealed extreme individual variations in the response among the eggs inoculated. The experimental design was modified, therefore, to attempt to determine the cause of the observed variations so that a suitable assay system and an alternative method of cultivation could be developed. To attempt to identify a possible source of the variation, embryonated eggs from a number of different chicken lines, both from out-bred lines and highly inbred lines were examined three days after inoculation with E. histolytica.

The eggs from the various chicken lines differed in at least two respects with regard to their susceptibility to E. histolytica: (1) the number of embryos from which E. histolytica could be recovered and (2) variation in the number of trophozoites recovered from individual positive eggs. Frequently, such variable infection responses in embryonated eggs can be explained by a number of well defined causes. Some of these known factors, however, did not appear to apply here. The hens from all flocks were fed antibiotic-free food and were free from pleuropneumonia-like organisms. The inbred flocks from SPAFAS were free of all pathogenic agents, including AVL viruses. The flocks from Truslow Farms (the out-bred flocks) were free of cytopathic avian viruses, but some hens were known to shed AVL virus. We demonstrated, however, that AVL virus did not inhibit amoebal trophozoites. Seasonal variations in the percentages of embryonated eggs from which amoebae could be recovered were not noted. In instances where the laying hens underwent a moult, no significant change in the percentage of positive embryos was found. In addition, the resistance to E. histolytica observed in some of the eggs could not be explained by production of interferon in the developing embryonated egg inoculated with amoebae or in the presence of E. histolytica cross-reacting antibody transmitted transovarially from the hen.

Other possible mechanisms of resistance were considered. Yolk sac extracts were examined for other anti-amoebal substances; none were found. Although some of the hens' sera were toxic for amoebal trophozoites in vitro, no correlation of this toxic property and the resistance of the embryonated eggs could be made. Neither could the resistance observed be explained on the basis of humoral factors or the immune response of the embryonated eggs, as such responses do not occur in hen's eggs.

When eggs derived from trap-nested hens, serviced by a single cockerel, were examined for their susceptibility to E. histolytica, the distribution of susceptible and resistant embryos remained relatively constant over long time periods. In some instances, individual single-mated hens could be identified which consistently layed only completely resistant or completely susceptible embryonated eggs. All of the above observations led to the conclusion that the genetic background of the chickens from which the embryonated eggs were derived may play a major role in determining their resistance or susceptibility to E. histolytica (Strain 458). Once highly sensitive and highly resistant birds were identified, it was possible to use brother-sister matings to select for susceptibility or resistance to E. histolytica, and to develop small flocks from these birds for use in genetic studies. At present, these flocks are under development, the F_1 generations are in production and the F_2 generations are almost ready for back-cross experiments to start.

Sensitive embryos were found most frequently in the out-bred birds. The results from the inoculations of eggs derived from these flocks suggest that susceptibility may be a dominant trait and that there may be some linkage with the C/A, COFAL-positive traits of these birds.

The inbred flocks used were developed for use in avian leukosis research. They were classified as C/O, COFAL negative, AVL virus sensitive. The relative resistance of the MR-41 embryonated eggs used in the trap-nest experiments suggests that in selecting for these traits, resistance to E. histolytica was selected for simultaneously.

It is expected that the genetically stable E. histolytica sensitive and resistant flocks developed on the basis of the findings reported here should provide useful tools for studies of 1) possible mechanisms responsible for these traits in the absence of known immune responses; 2) how the genetic constitution of a host may change the amoebae; 3) how trophozoites may be altered by multiple passages in resistant and sensitive hosts. The sensitive flocks may be useful, also, in providing embryonated eggs for virulence and infectivity assay systems for E. histolytica.

If a genetic basis is the correct explanation of the resistance and susceptibility observed in ovo, it seemed reasonable that it should be possible, also, to demonstrate these traits in vitro. Our recent studies have demonstrated that there is a direct relationship between susceptibility to amoebal trophozoites in ovo and in vitro and that the differences in susceptibility observed in ovo were maintained at a cellular level. Dose-response curves utilizing chick embryo fibroblasts (CEF) derived from individual eggs showed marked differences in the degree of lysis observed in cultures derived from sensitive and resistance eggs. CEF prepared from embryonated hen's eggs susceptible to E. histolytica were completely lysed 3-5 hours after inoculation with 5,000 axenically grown H-458 strain trophozoites. In contrast, CEF derived from resistant eggs remained in good condition for 7-plus hours after inoculation of amoeba, and viable CEF could be recovered after 24 hours.

These differences in cytopathic effects observed between susceptible and resistant primary CEF were maintained even after 10 tissue culture passages. It was essential that viable trophozoites were in direct contact with the CEF for the cytopathic effect to occur. The use of susceptible and resistant lines of CEF which we have developed may offer a model, at a cellular level, for studying the mechanisms of genetic control of susceptibility of chickens to E. histolytica.

Role of viruses of E. histolytica in humans: A sero-epidemiological approach is being employed for these studies. We are attempting to determine the antibody levels, if any, for the beaded and the icosahedral viruses in amoebiasis patients with amoebic colitis, amoebic liver abscess, and in carrier populations. Serial serum samples, before and after treatment, have been obtained from a number of these patients. Viral antigen and specific hyperimmune sera to two strains of the beaded virus have been prepared and used to standardize complement-fixation and agar-gel diffusion tests. These reagents will be used also in ELISA and RIA tests. Purification of the icosahedral virus in quantities sufficient to use in serologic screening has not been achieved, despite our use of all the usual concentration and purification techniques and the application of isoelectric focusing and isotachophoresis to this problem.

Preparation of viral antigens and specific hyperimmune sera from other strains of beaded virus will be made to check for group and type specific antigens. In addition to their use as standard reagents, these materials should prove useful, also, as a probe to study the virus interaction with its host amoeba.

Development of pathogenic variants of E. histolytica with difference immunologic characteristics: Serial passages of protozoa in embryonated eggs have been used in the past to select towards virulence for chicken embryos. This technique has been used also, to produce attenuated strains of protozoa for inoculation into other hosts. Although virulence and antigenic changes were seen after passages of amoebae in randomly selected eggs, interpretation of virulence changes and possible accompanying antigenic changes should be made simpler using data we are accumulating from passages in uniformly susceptible or resistant eggs. In addition, use of uniformly susceptible eggs and tissue cultures should provide for easier interpretation of serum neutralization tests and antibody induced antigenic alterations of E. histolytica antigens.

Significance to Biomedical Research

The study of viruses in parasitic protozoa, many of which are endemic in the United States and elsewhere, may offer useful information on the role, function, and possible manipulation of extrachromosomal elements which may lead to control and treatment of the protozoa. Development of susceptible and resistant sources of avian tissue will allow studies of host-parasite interactions at a cell membrane, intracellular and subcellular level, which may help our understanding of the success or failure of protozoal infections in the intact animal.

Publications

Jaouni, K.C.: Entamoeba histolytica: Genetic control of susceptibility in avian tissues. I. In ovo studies. Experimental Parasitology, In press.

Other

Member, Joint Board on Science and Engineering Education, Washington Academy of Science.

Committee member, Gifted Science Project, Department of Curriculum and Instruction, Montgomery County Public Schools

Coordinator, Rocky Mountain Laboratory Seminars.

International Committee on Taxonomy of Viruses, Subcommittee on Bacterial Viruses.

Presented paper "Correlation of susceptibility to Entamoeba histolytica trophozoites in ovo and in vitro", at the 29th Annual American Institute of Biological Sciences, Athens, Georgia.

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PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of Viral Antigens in Virus-induced Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The initial objectives of this project were to use human adenoviruses and adenovirus - SV40(Ad2-240) recombinants as tools to study the genetics of DNA tumor viruses, to define the role of viral genes and viral antigens in viral oncogenesis and to study the biology of Ad2-SV40 recombinants. Due to the lack of proper containment facilities in NIAID between January 1973 and May 1978, there has been a 5 year disruption (see past reports) in the major thrust of this project. During this interval, a study of Ad2-SV40 recombinants associated tumor induction by these agents with the incorporation into the adenovirus 2 chromosome of a specific segment of SV40 DNA. To begin to understand the mechanism by which this SV40 DNA segment conveys oncogenicity to these recombinants, it was necessary to understand why Ad2 was nononcogenic for hamsters. Since Ad2 will transform hamster cells in tissue culture, we initiated a study of the oncogenic properties of Ad2 transformed cells. The results thus far lead us to suspect that unrecognized host immune mechanisms in the hamster reject Ad2 transformed cells and transformed cell-induced tumors. Such a concept suggests that the presence of a specific segment of the SV40 genome might induce functions in hamster tumor

cells that interfere with the rejection process. Future studies will be directed toward more carefully defining these concepts.

Project Description

Major Findings: Association of tumor induction by nondefective Ad2-SV40 recombinants with a specific segment of SV40 DNA

Studies of defective Ad-SV40 hybrid populations demonstrated that the incorporation of large segments of SV40 DNA into oncogenic or nononcogenic adenoviruses enhanced the tumor inducing capacity of the recombinants. The availability of nondefective Ad2-SV40 recombinants containing overlapping regions of the early SV40 genome provided an opportunity to determine the ability of small segments of the SV40 genome to influence the oncogenicity of nondefective Ad2-SV40 recombinants. *In vitro* assays of the transforming capacity of these agents required the use of ultraviolet (UV) light inactivated stocks to retard cytopathic effects. These studies failed to demonstrate significant differences in the transforming capacity of nonhybrid Ad2 and the $Ad2^{+}ND_2$, $Ad2^{+}ND_4$ and B55 hybrids carrying 54, 87 and 87 percent respectively of the early SV40 genome. As an *in vivo* corollary to these experiments, newborn hamsters were injected with inactivated nonhybrid Ad2 and the $Ad^{+}ND2$, $Ad2^{+}ND_4$, and B55 recombinants.

The nondefective hybrid, $Ad2^{+}ND_4$, which contains the segment of the SV40 genome between map position 0.11 and 0.59 induced tumors in 9 to 15% of hamsters after inactivation by UV light. Ad2 and the $Ad2^{+}ND_2$ hybrid which contains the segment of the SV40 genome between map position 0.11 and 0.43 did not induce tumors after UV light inactivation. These results show that the incorporation of a specific segment of SV40 DNA into the Ad2 genome can alter the pathogenesis of this virus by rendering it oncogenic for hamsters. The segment of the SV40 genome between 0.43 and 0.59 which is associated with the ability of the $Ad2^{+}ND_4$/B55 hybrids to induce serologically reactive SV40 T antigen is required for tumor induction.

Host response to adenovirus 2 transformed hamster embryo cells

The finding that a segment of SV40 DNA, by recombining with the nononcogenic Ad2 genome, conveyed tumor-inducing properties to the $Ad2^{+}ND_4$ raised questions about the mechanism by which this change to oncogenicity was effected. Others have shown that Ad2 transformed rat cells were incapable of establishing tumors in immunocompetent rats. Since there appears to be little or no difference between the *in vitro* transforming efficiency of the $Ad2^{+}ND_2$ and $Ad2^{+}ND_4$ recombinants and nonhybrid A2, it seemed reasonable to assume that the change toward oncogenicity could be a reflection of the manner in which cells transformed *in vivo* by hybrid and nonhybrid virions interacted with the host immune system. As there was little data about the tumor inducing capacity of Ad2 transformed hamster cells, it was necessary to examine this problem before studies examining the influence of the SV40 genome on tumor induction by the recombinant could be considered.

Ad2 inactivated by UV light was used to transform LSH hamster embryo cells. Evaluation of transformed cell lines produced by Ad2 (strain adenoid 6) or isolates of Ad2 obtained from children in Washington, D. C., or West Bengal, India, showed that 13 of 15 lines induced tumors when injected into newborn inbred LSH or randomly bred NIH hamsters (10^7 cells/ hamster) with a median tumor incidence in syngeneic newborns of 65% (range 8 to 100%). Six out of 6

of these cell lines did not induce tumors when 10^7 cells were injected s.c. into weanling animals over 21 days old. In contrast to the Ad2-transformed lines, similarly derived cell lines transformed by Ad12 or SV40 uniformly produced tumors in weanlings following s.c. inoculation of 10^7 cells. Transplantation of tumors from animals injected as newborns with Ad2-transformed cell lines to other newborns was readily accomplished in 639 of 640, (99.8%) inoculated newborns. However, only 53 of 467 (11.3%) weanling hamsters challenged with these tumor lines developed neoplasms. Each of these Ad2-transformed lines contained Ad2 T antigen detectable by complement fixation or immunofluorescence. None of 6 lines contained Ad2 by Sendai fusion with human embryonic kidney (HEK) cells or other contaminating agents by a variety of assays. The difference in oncogenicity in weanling hamsters of Ad2-transformed cells compared to Ad12 - and SV40-transformed cells was not related to the dose of tumor injected or to differences in in vitro growth properties of the cell lines (e.g., doubling times, saturation densities, growth in spinner, or colony formation in soft agar). The tumor inducing capacity of Ad2 newborn tumor lines for newborn and weanling hamsters was not related to the dose of tumor injected. With the two tumor lines tested, weanling rejection of newborn tumor transplants could not be overcome by inocula containing 25 times the usual dose of tumor suspension. These same two newborn tumor lines produced progressively enlarging neoplasms in 17-77% of syngeneic weanling hamsters which had been thymectomized as newborns. The lack of oncogenecity of hamster cells transformed in vitro by Ad2 supported our speculation that the SV40 genome in cells transformed in vivo by the Ad2$^+$ND$_4$ recombinant could be altering immunological determinants involved in tumor cell rejection.

Viral DNA sequences and gene products in hamster cells transformed by Ad2.

Complementary strand-specific adenovirus DNA of full length or from endonuclease Bam HI fragments was used as a probe to estimate the fractional representation and abundance of viral sequences in five hamster cell lines Ad2HE1-5 transformed with UV-inactivated Ad2. The fraction of the viral genome present in the five transformed cell lines varied from 44% in the Ad2HE5 cell line to 84% in the Ad2HE3 cell line. The number of viral DNA copies per diploid cell equivalent ranged from 1.8 in the Ad2HE1 line to 7.1 in the Ad2HE4 line.

In vivo labeling with ^{35}S-methionine followed by immunoprecipitation with an antiserum against Ad2 early proteins revealed viral-specific polypeptides with molecular weights of 42,000 to 58,000 in extracts from all five hamster cell lines. Several other early viral polypeptides were detected in some of the Ad2 transformed hamster cell lines.

Studies are underway to determine the specific regions of the Ad2 genome that are contained in the Ad2HE1 line of Ad2 transformed hamster embryo cells and to determine which of the messenger RNA's produced in these cells are coded for by these Ad2 DNA containing regions. Preliminary results indicate that RNA coded for by the early region on the left hand end of the right strand (the "transforming" region) is present; in addition, RNAs representing regions of the Ad2 genome transcribed late during infection (i.e. after viral DNA replication begins) are also present but appear in patterns not typical of RNA from cells productively infected with Ad2.

Role of the hamster cellular immune response in Ad2 transformed cell oncogenesis

The tumor-inducing capacity of two Ad2 transformed hamster cell-induced lines (Ad2HTL3 and Ad2HTL6) was tested in immunocompromised hosts. An average of 35.2% of neonatally thymectomized, syngeneic, weanling hamsters developed progressively enlarging tumors when injected subcutaneously with tumor suspensions prepared from these lines, while no tumors were observed in normal, syngeneic, weanling hamsters challenged with the same inocula. The susceptibility of neonatally thymectomized hamsters to tumor challenge was directly related to the degree of immunosuppression observed following thymectomy as indicated by the amplitude of the *in vitro* response of whole blood cultures to concanavalin A. Pretreatment of thymectomized weanlings with syngeneic adult lymphoid cells resulted in a significant reduction in tumor susceptibility ($p = 0.03$). These findings suggest that the maturation of a thymus-dependent cell-mediated immune response in hamsters during the first 21 days of life results in the rejection of Ad2-transformed cells.

Developmental hamster immunobiology related to tumor rejection in the Ad2 transformed hamster cell system

Transplantation of the Ad2HTL6 newborn tumor line to suckling hamsters 1-15 day old demonstrated that maturation events in the hamsters immune system between 6 and 7 days were responsible for tumor rejection. Very little data has been published concerning the ontogeny of the hamster immune response; however, based on data from other species, it has been predicted that hamster thymus and spleen cells should respond to T cell mitogens within a few days of birth and that suckling hamsters should be able to reject skin allografts at 8 days of age. To begin to evaluate the development of cellular immune responses in hamsters, cells from bloods and spleens from different aged animals were tested for their ability to respond to the mitogen conconavalin A (ConA) and to respond in the mixed lymphocyte reaction. The results of these studies indicate that hamster spleen cells begin to respond to ConA by age 5 days and attained 100 percent of adult type responses by age 15 days. Cells in the blood did not respond to ConA until age 25 days.

Hamster spleen cells did not begin to respond in the mixed lymphocyte reaction until 18 to 25 days. To determine if the lack of ConA responsiveness of hamster spleen cells before age 5 days was due to T cell immaturity or immaturity of another cell component which was required for this response, X-ray inactivated adherent and nonadherent (to distinguish between lymphocytes and macrophages) adult peritoneal exudate cells were mixed with spleen cells from 3 day old hamsters and the mixtures tested for their ability to respond to ConA. These results showed that, in the presence of populations of adherent peritoneal exudate cells, 3 day old hamster spleen cells readily responded (stimulation indices of 13 to 35) compared to spleen cells alone (stimulation index of 1 to 2) or spleen cells mixed with nonadherent cells (stimulation index of 1 to 3). These results imply that immaturity of cell populations which cooperate with lymphocytes in the response to ConA are most likely responsible for the apparent immaturity of this reaction in hamsters less than 5 days old.

As an *in vivo* corollary to the evaluation of immune cell responses to mitogen and foreign antigens, the inflammatory response to tumor development in newborn, weanlings and weanlings thymectomized during the first 24 hours of life

was examined histopathologically in animals injected with suspensions of Ad2HTL3 and Ad2HTL6 tumors. In newborn animals multiple tumor nodules developed within 5 days and were quickly enclosed by highly vascular connective tissue. New capillaries were seen entering these nodules. During the first few days inflammatory cells consisted mainly of polymorphonuclear leukocytes with some lymphocytes and histocytes around the forming nodules. In sections with cells invading the surrounding connective tissue, there was no inflammatory response. The response to tumor development in thymectomized weanlings was similar to that observed in newborns. In normal weanlings, tumor nodules developed early but were regressing by 5 days. Some new vessel formation was present during the first few days of tumor nodule development but this was less impressive than the intense vascular response noted in newborns. Inflammatory cells consisted of polymorphonuclear leukocytes during the first day followed by the rapid appearance of an intense lymphohistiocytic infiltrate. After 7 days, the tumor nodules consisted of mostly granulation tissue with many multinucleated giant cells and focal calcification.

Immunogenicity of Ad2 transformed hamster cells and transformed cell-induced tumor lines

To determine the possible role of Ad2 transplantation antigen (TSTA) in the rejection of tumors transplanted to weanling hamsters, several types of studies are underway. An assay has been developed for Ad2 TSTA using an Ad2 tumor line (Ad2HTL1) which had been adapted to grow in adult hamsters. Immunization with Ad2 significantly protected (defined as a resistance index *RI) greater than 10) hamsters against a challenge with Ad2HTL1. Immunization with SV40 did not convey significant protection (RI between 1 to 10) in these experiments. This assay is being used to study the immunogenicity of Ad2 transformed cell lines and newborn and weanling tumor lines developed from these transformed cells. Results thus far indicate that 3 Ad2 transformed cell lines (Ad2HE1, Ad2HE3, Ad2HE6) and the tumor lines established from them (Ad2HTL1, Ad2HTL3, Ad2HTL6) contain a common Ad2 TSTA which during immunization with x-irradicated cells conveys protection to hamsters challenged with Ad2HTL1. Differences in RI did not correlate with the differences in the transplantability of the newborn tumor lines to newborn and weanling hamsters. Adult hamsters that had rejected tumors induced by viable Ad2HTL3 and Ad2HTL6 cells were also protected when challenged with Ad2HTL1. These results show that whether they are accepted or rejected by weanling hamsters,each of these lines contains detectable Ad2 TSTA. Such findings appear to exclude the possibility that certain tumor lines can be transplanted to older animals because they lose detectable Ad2 TSTA. To determine whether a reduction in the concentration of Ad2 TSTA is responsible for the transplantability of certain newborn tumor lines to adults, the amount of immunizing antigen in suspensions of different tumors is being evaluated.

Difference in the transplantability of virus-induced neoplastic cells in inbred hamsters.

Tumor cells behave like normal cells in that they are usually rejected when transplanted as allografts to histocompatible hosts. Since hamsters are suspected to lack resistance to transplantable tumors of viral origin, we have been studying the transplantability of our transformed cells and tumor lines to different strains of inbred hamsters. Eleven Ad2, Ad12 and SV40 transformed LSH cell lines and LSH tumor lines established from them are being transplanted to syngenic LSH hamsters, CB hamsters which are histo-

compatibility complex (MHC) disparate, and PD4 hamsters which are MHC identical but disparate at minor H loci. Initial findings show that Ad2 transformed cells produced tumors only in newborn LSH hamsters. Ad2 tumor lines (Ad2HTL1, Ad2HTL3) adopted to grow in LSH adults by serial passage in neonatal hamsters produced tumors readily (TPD_{50} $10^{2.3}$ to $10^{4.9}$/0.2 ml of tumor suspension) when transplanted to LSH adults. These same lines produced tumors inefficiently (TPD_{50} $10^{0.0}$ to $10^{2.2}$/0.2 ml) when transplanted to adult CB hamsters and of intermediate efficiency (TPP_{50} $10^{1.4}$ to $10^{3.7}$/0.2 ml) when transplanted to PD_4 hamsters. When 10^6 to 10^7 Ad12 transformed LSH cells were inoculated into adult LSH hamsters 84% developed tumors. When the same cell doses were injected into CB hamsters only 33% developed tumors. Tumor lines established in LSH hamsters from Ad12 transformed cells produced tumors in a pattern similar to the Ad2 tumor lines when transplanted to adult LSH, CB, and PD_4 hamsters. When 10^6 and 10^7 SV40 transformed LSH cells were injected into adult LSH and CB hamsters, 100% and 97% respectively developed tumors. SV40 tumor lines produced tumors with equal efficiency in all 3 hamster strains. Thus, our Ad2 induced neoplastic LSH cells produced tumors only in immunoimmature syngeneic LSH hamsters; our Ad12 induced neoplastic LSH cells produced tumors efficiently in both immunoimmature and immunomature syngeneic LSH hamsters but produced tumors less efficiently in allogeneic hamsters; our SV40 induced neoplastic LSH cells produced tumors efficiently in both syngeneic and allogeneic hamsters.

Current ideas about the role of TSTA and allograft rejection of viral induced neoplasms do not account for the finding above. In this regard, we are considering the possibility that the differences in the transplantability of these various cell lines are a reflection of the unrecognized manner in which tumor and allograft determinants on the surface of hamster cells are altered during transformation events by a particular viral agent. Studies are underway to further clarify these findings.

Significance to Biomedical Research

The results of our recent studies impinge on tumor immunology and the possible role of antigenic determinants on cell surfaces in determining the outcome of viral-induced malignancy. The availability of well-characterized tumor lines, which are transplantable to both immunoimmature and immunomature hamsters and tumor lines, which are transplantable to immunoimmature but are consistently rejected by immunomature animals provides a useful system for identifying and studying the maturation of the functions of the hamster cellular immune system responsible for the rejection of viral-induced tumors. The central theme of tumor virology is the concept that viruses induce the malignant state (i.e., the ability to produce tumors in a susceptible host) in cells by a process called transformation. A number of studies (including our own work with the nondefective Ad2-SV40 hybrids) with different agents have associated transformation and tumor induction with the functioning of a specific region of the viral genome and in some cases, with a specific gene product. Paradoxically, many virus-transformed cells which possess the properties ascribed to the transformed state fail to produce tumors when injected into either syngeneic immunomature or immunoimmature hosts. The reasons for this lack of transformed cell oncogenicity for a host that should be susceptible are poorly understood. Our studies with viral transformed hamster cells provide data

which pertain to this question. At this juncture, we interpret our results with the Ad2 and SV40 transformed LSH hamster cell system as suggesting that, during the process of transformation, antigenic determinants on cell surfaces in addition to TSTA are altered. Some of these determinants are associated with graft rejection by allogeneic hosts; others are associated with the recognition and rejection of tumor cells by syngeneic hosts. An understanding of these cell surface modulations and the role of the viral genome in the alteration of their function would be a significant step in understanding the mechanism by which viruses convert normal cells to malignant ones.

Publications

Lewis, A. M., Jr. Defective and Nondefective Ad2-SV40 Hybrids. Prog. Med. Virol. 23:96-139, 1977.

Patch, C. T., Levine, A. S., and Lewis, A. M., Jr. The Adenovirus-SV40 Hybrid Viruses. Comprehensive Virology. In press.

Johansson, K.; Persson, H.; Lewis, A. M., Jr.: Petterson, U.; Tibbetts, C. and Philipson, L. Viral DNA Sequences and Gene Products in Hamster Cells Transformed by Adenovirus Type 2. J. Virol. In press.

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PHS-NIH
Summary Statement
Laboratory of Streptococcal Diseases
October 1, 1977 to September 30, 1978

Research Progress

Erythrogenic Toxin of a Non-lysogenic Group A Streptococci

The identity of the toxin (previously identified as B) that is produced by non-lysogenized strain 64x402, was confirmed by a line of identity with a standard isoelectrically-purified B toxin, in Ouchterlony tests with monospecific B antitoxin. Polyacrylamide gel electrophoretic comparison also identified B toxin in the 64x402 material, although some contaminating proteins were also present. Although C toxin was produced by vegetative infection of 64x402 with phage AT203U (from a C-producing strain), similar infection of this or some non-toxinogenic strains of streptococci of Group A and C by phages from other strains producing toxins A, B, or C, failed to produce any detectable toxin. No plasmids were detected in 64x402, and its DNA failed to transform a competent strain of Group H streptococci to toxin production although a streptomycin resistance marker was integrated and expressed. The location and nature of the genetic coding for B toxin remain unknown, but will be further explored by use of different recipient cells and other methods for transfer of DNA. (Colon Whitt, Cole)

Viruses of Spiroplasmas

The high, if not universal, prevalence of 1 or more viruses in spiroplasmas was shown by electron microscopy (93% of 56 strains positive) and by plaquing on 25 spiroplasma lawns (100% of 29 strains positive). Not all strains are positive on all lawns, the numbers of plaques produced by a given wash differ from lawn to lawn, purified viruses SVC3/608 and SVC3/SMCA show host-range differences, and some strains appear to be self-inducible--all suggesting mechanisms of immunity, restriction, and modification that have yet to be elucidated. Strains tested, in addition to those previously available from citrus stubborn disease, corn stunt disease, and the suckling mouse cataract agent, now include other isolates from ticks, bees, cactus, lettuce, and flowers. The virus or viruses responsible for plaquing were not identified in all instances, but a combination of one bee spiroplasma wash spotted on a lawn of another bee spiroplasma produced, for the first time, plaques shown to be caused by SVC1. This rod-shaped virus was propagated in broth cultures of the same lawn strain, and purified by density gradient centrifugation. It appears to contain DNA and characterization

is in progress. Further studies of the previously purified polyhedral virus, SVC3/608, determined its adsorption rate (1.3×10^{-9} cm^3/min), latent period (21 hr), burst size 26-30 pfu), and K value of homologous antiserum (880). Its double stranded DNA has a G+C content of 27 mol. %, and contains both cyclic permutation and terminal repetition of nucleotide sequences and can transfect host cells. The availability of 2 viruses as probes makes possible more extensive investigations of virus-host interactions in wall-less prokaryotes. (Cole, Mitchell, Liss, Garon(LBV), Jablonska, Popkin)

DNA Uptake in Microbial Genetic Transformation

The 3 steps that lead to genetic transformation in bacteria that require divalent cations are (1) binding or attachment of the DNA to specific surface receptors, (2) entry of the DNA into the cell's cytoplasm, and (3) degradation of a good part of the supplied DNA to acid-soluble products. This last step may be associated with entry. Previously, it was shown that step (1) was independent of cations when homologous DNA was used. This observation can now be explained. It appears that "purified" DNA preparations contain, tightly associated with them, a protein that can act as a divalent cation at pH 6.5 (a basic protein). When the protein is removed, the "naked" DNA no longer binds to competent cells unless certain divalent cations are provided. Of the cations tested, Ca, Zn, Fe, and Cu supported step (1). Step (2) was stimulated only by Ca, whereas step (3) was stimulated to various degrees by Ca, Mn, Co, Mg, Fe, Cd, and Ni. None of these, acting singly or in combination, were capable of reversing the inhibition of steps (1) and (3) caused by certain chelating agents (EDTA or EGTA). The reason for this is not understood. However, progress is being made in understanding now DNA is transported into competent cells and what factors influence it. (Ranhand)

Bacterial Lipoteichoic Acid

Lipoteichoic acid (LTA) of gram-positive bacteria, aside from functioning in part as an intermediate in synthesis of cell wall teichoic acid, has no known role but is a constant cellular component. Our recent studies confirm last year's mention of changes in total cellular LTA during growth, showing an increase through exponential phase, followed by a fall in stationary phase. (Total level and the degree of change vary from species to species.) However, LTA is measured as high molecular weight Peak I material (Sepharose 6B column), and might be undetected if degraded enzymatically or otherwise to a lower molecular weight. To test if this might happen during the protoplasting used to obtain cellular fractions of S. aureus, 32P-labelled LTA was added to the start of the procedure and counts made of the several fractions obtained. Most of this LTA was recovered in the periplasm-

cytoplasm fraction, but 90% had been degraded to Peak II or smaller molecular weight. The small amount of LTA found with the membrane fraction was degraded to a similar extent. Labelled LTA added to previously prepared mesosome or membrane fractions was adsorbed to some extent (better by membranes). Lack of evidence of exchange, in the mesosome fraction, with the endogenous LTA suggests that the latter is sequestered within the mesosomal vesicles where it is protected from enzymatic attack and thus accounts for the recovery of the bulk of LTA from the mesosomal fraction. (Huff)

Extraction of LTA from subcellular fractions of S. aureus at several stages of growth showed that, in each growth phase, LTA detectable as Peak I material was found primarily in the mesosome fraction, to a small extent in the membrane fraction, and never in cytoplasm, periplasm, or extracellular culture fluid. Periplasm, cytoplasm, and membranes contained Peak II material (never found in meososmes) as well as an unidentified glycerol-phosphate compound of unknown significance. An optimum procedure (hot 80% aqueous phenol, DNAase, RNAase, Sepharose 6B chromatography) isolated LTA representing 2% of the dry weight of the staphylococcal cell. This Peak I material (not previously analysed in detail) contained no protein, less than 0.3% nucleic acid, no amino sugars, a single amino acid (D-alanine), 6 fatty acids (C15-C24), 3.75 micromol. glycerol, and 3.51 micromol. phosphate. Carbohydrate analysis is in progress. This material, lacking protein, is not immunogenic and prior attempts to use it or other preparations to produce specific LTA-antisera were unsuccessful. However, LTA extracted with cold 80% aqueous phenol and used with adjuvants in a prolonged immunization schedule, has now produced rabbit antiserum reacting in a single precipitin line with homologous antigen and deacylated LTA. The IgG fraction is now being used in immunoelectron microscopy to attempt to localize LTA in thin sections of the intact cell--in order to verify directly all other evidence that points to the mesosome as the intracellular site of LTA in Staphylococcus aureus. (Theodore, Fraser:LIG)

Bacteriophage Nucleic Acids

The nucleic acid of temperate phage N3 of Haemophilus influenzae Rd9 (N3) was shown to be a double-stranded linear DNA molecule (MW 26 x 10^6 daltons) with "sticky" ends and a G+C content of 43 mol. %. Digestion with EcoR1 restriction endonuclease and agarose gel electrophoresis distinguished 6 fragments of molecular weights 7.05, 5.67, 4.48, 4.30, 2.71, and 2.16 x 10^6 daltons, from which a circular physical map of the genome was constructed. This DNA is tranfecting, and its structure resembles that of lambda phage DNA--although the circular duplexes formed in neutral salt solutions are not

thermally reversible as they are in lambda. (Jablonska; DeFilippes and Garon, LBV)

The nucleic acids of several apparently identical phages of salivary origin which can lysogenize a Group H streptococcus (WE4), was demonstrated to be linear double-stranded DNA of MW 24.6×10^6 daltons, with a G+C content of 44.4 mol. %. By denaturation and reannealing, and treatment with Exonuclease III followed by reannealing, this DNA was shown to be circularly permuted and terminally repetitious. Although coliphage T2 DNA is permuted and coliphages T3 and T7 DNAs have terminally repeated sequences, the combination of these has been thought to be rare, occurring notably in the DNA of Salmonella phage P22. It has now been seen also in the DNA of spiroplasma virus SVC3 (See Project No. Z01 AI 00007-4) and may be more common than suspected. Restriction endonuclease analysis of the phage DNA is in progress. (Moynet; Garon and DeFilippes, LBV)

Administrative, Organizational, and Other Changes

The scope of research remains as before, involving 6 projects concerned with streptococci, staphylococci, mycoplasmas and spiroplasmas, streptococcal bacteriophages, viruses of mycoplasmas and spiroplasmas, microbial genetics lipoteichoic acids, viral nucleic acids, and related subjects. Dr. Elzbieta Jablonska (Visiting Fellow) returned to Poland in April. Dr. Daniel Moynet (Visiting Fellow) had his Fellowship renewed to February 1979. Both Fellows have worked intensively on characterization of different phages and their nucleic acids. In October 1977, Dr. Alan Liss (Senior Staff Fellow) arrived, and has strengthened our efforts to understand mycoplasma and spiroplasma viruses. Dr. David L. Williamson (Visiting Investigator) was assigned through the Intergovernmental Personnel Act from the State University of New York at Stoneybrook and arrived in February 1978. He has been actively engaged in collaborative studies to clarify the nature, serology, and virology of spiroplasmas. In July, Mr. Clyde Tapp (Biol. Lab. Tech.) retired after 18 years of governmental service, of which 13 were spent in the Laboratory. Ms. Deborah L. Hartman (Biologist) transferred to LPD in July. Dr. Gary Calandra is completing an assignment in infectious diseases, on detail to LCI.

Although no new directions nor expansions can be anticipated, in view of current restrictions on the overall position ceiling, it is to be hoped that at least the former level of 17 positions in LDS can be restored in the near future. In respect to the Institute's name and mission, it would appear important to maintain, and possibly expand, the availability of bacteriological expertise at Bethesda--which, aside from contributing to knowledge of the structure and function of prokaryotic microorganisms and their viruses, can supply frequently requested advice and consultation on the subject area.

Honors and Awards

Roger M. Cole

Invited participant (Symposium Speaker on Spiroplasma Viruses: Session Chairman) II Meeting of the International Organization of Mycoplasmologists; Freiburg, Germany, Aug. 28-Sept. 2, 1978.

Invited participant (Workshop Session Speaker on Spiroplasma Viruses), 4th International Congress for Virology, The Hague, The Netherlands. Aug. 30-Sept. 6, 1978.

Invited participant, 7th International Symposium on Streptococci and Streptococcal Disease. St. Anne's College, Oxford, England. Sept. 12-Sept. 15, 1978.

Member, Editorial Board, Infection and Immunity.

Jon M. Ranhand

Invited participant, IV European Meeting on Bacterial Transformation and Transfection. York, England. Aug. 28-Sept. 1, 1978.

Alan Liss

Invited participant (Symposium Speaker on Biology of Ureaplasma: Progress and Prospects) Annual Meeting of the American Society of Microbiology, Las Vegas, May 14-18, 1978.

Invited participant (Workshop Session) 4th International Congress for Virology, The Hague, The Netherlands. Aug. 30-Sept. 6, 1978.

Presented invited seminar on Mycoplasma and Spiroplasma Viruses, Kansas State University. May 12, 1978.

Visiting Foreign Scientist, The Juselius Foundation, Tampere, Finland. August 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI00001-17 LSD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Biochemical Studies on Staphylococcal and Streptococcal Membranes, Cell Walls, and Mesosomal Vesicles.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. Huff	Scientist Director	LSD, NIAID
Other:	T. S. Theodore	Res. Microbiologist	LSD, NIAID

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this project is to study the biochemical processes involved in the growth of bacterial cell wall and membrane and the coupling of these processes to DNA replication during cell division. The present goal is to determine the functional role of lipoteichoic acid in Staphylococcus aureus and other Gram-positive bacteria. Lipoteichoic acids are polymers of glycerol phosphate found intracellularly closely associated with plasma membranes and with membranous bodies known as mesosomes. One of the polyglycerol phosphates, lipoteichoic carrier, has been implicated by others in the synthesis of cell wall teichoic acid. This material is a minor component of the lipoteichoic acid fraction. The function of the remainder of the lipoteichoic acid (LTA) is not known and this is the area of current interest. Present studies are directed at: 1) relating LTA content to cellular growth; 2) location of LTA within Gram-positive bacteria other than S. aureus; 3) location of LTA within the mesosome.

Project Description:

Lipoteichoic acids are antigenic glycerol phosphate polymers found intracellularly closely associated with membranous organelles known as mesosomes in Staphylococcus aureus. One of the poly-glycerol phosphates, lipoteichoic carrier, has been implicated in the synthesis of cell wall teichoic acid. This material appears to be a minor component of the lipoteichoic acid fraction. The purpose of this study has been to determine the function of the bulk of the intracellular lipoteichoic acid (LTA) which is not implicated in the assembly of teichoic acid. Specifically, efforts have been directed at: 1) relating LTA content to cellular growth in S. aureus and several Gram-positive organisms; 2) location of LTA within bacteria other than S. aureus; 3) location of LTA within the mesosome of S. aureus; 4) determining enzymatic destruction of LTA is S. aureus.

Stationary (18 hr) cells of S. aureus strain Lafferty have low levels (0 to 0.1% of the dry weight of cells) of LTA (measured as peak I material off Sepharose 6B). Upon dilution into fresh media and incubation at 37 C, there is a rapid increase in the LTA level to 1.3%. Then as the cells go out of exponential growth, the LTA level drops back to 0.1% over a 2 hour period. Similar results were obtained with S. aureus ATCC 6538P except that the drop in LTA started one hour before the end of exponential growth. In S. faecalis ATCC 9790 the intracellular level of LTA in stationary cells was 0.6% of the dry weight. Upon dilution into fresh media, there was a rapid 4-fold increase in LTA followed by a drop back to stationary cell levels as the cells went out of exponential growth. The level of LTA in Bacillus licheniformis ATCC 9945 continued to increase for 3 hours from 0.3% to 1.6% followed by a rapid drop back to starting levels as cells went out of exponential growth. In B. subtilis ATCC 6051 the LTA increased slowly from 0.2% to 0.6% over a 3 hour period after inoculation of stationary cells into fresh media. In S. pyogenes (56x188) LTA increased from 0.3% to 0.6% during 4 hours of growth. Within the bacterial strains studied the LTA level during growth ranges from 20-fold changes in S. aureus to 2-fold changes in S. pyogenes.

Controversy exists regarding the intracellular location of lipo-teichoic acid. Our own studies have shown the almost exclusive localization of LTA in the mesosomal fraction of S. aureus. Our attempts to localize the LTA in S. faecalis, S. sanguis, and S. mutans have been hampered by poor recovery of LTA. This poor recovery could have been due to losses of LTA during protoplasting and preparation of fractions due to enzymatic destruction. We wished to better understand the chemical nature of the association of LTA and the mesosome in S. aureus and to test for enzymatic destruction of LTA in S. aureus and streptococcal strains. P^{32}-LTA (Peak I) from S. aureus 6538P was incubated with cells of the same organism during protoplasting. LTA was recovered (94%)

in the periplasm fraction as unaltered peak I. About 1% of the LTA was found associated with the mesosomal fraction. Separate incubation of P^{32}-LTA with mesosomes and membranes showed a time dependent absorption of added P^{32}-LTA to mesosomes which ceased when the absorbed LTA had reached 0.5% of the total mesosomal LTA. This indicates that added LTA cannot exchange with the bulk of the mesosomal LTA. Membranes absorbed twice as much P^{32}-LTA on a weight basis as the mesosome. The simplest explanation of these results is that the added LTA absorbed only to the exterior of the mesosome while the bulk of the LTA was located on the inside of the mesosome in an unbound form.

When P^{32}-LTA (peak I) was incubated with exponential cells of S. aureus 6538P during lysostaphin lysis, 61% of the counts were in the combined periplasm-cytoplasm fraction; 10% in the washed membranes and 5% in the mesosomes. Comparable figures for stationary cells of this strain were 43%, 10%, and 4%. Dialysis and rechromatography of the material from the cytoplasm-periplasm fraction of exponential cells on Sepharose 6B showed that 80% of the LTA was dialyzable; 10% was unchanged peak I and 10% peak II material. Peak II is a smaller molecular weight fraction of LTA which separates from the micellar LTA (peak I) on Sepharose 6B. Of the LTA found in the membranes from exponential cell lysis, 91% was dialyzable, 29% in peak I, and 8% in peak II. These results show that once the cells are lysed, added LTA becomes subject to enzymatic alteration to peak II and smaller molecular weight substances as well as being absorbed onto membranes in S. aureus. The good recovery of LTA from the various cellular fractions of S aureus in spite of the presence of destructive enzymes may be due to the inaccessibility of the LTA in the mesosomes to enzymatic attack.

These results show that lipoteichoic acid is either absent or present at very low levels in stationary or non-dividing cells. As cells go into exponential growth, the LTA rapidly increases to 1% to 2% of the dry weight. In S. aureus LTA is incorporated in the interior of mesosomes which arise from the membrane in the region of the septum prior to crosswall formation. Mesosomes may result from a nucleoid contraction process in which cell membrane is pulled into the cytoplasm via DNA-membrane attachment points. The mesosomes must play an active role in the biosynthesis of LTA, cell wall and membrane. Once exponential growth ceases LTA rapidly disappears, perhaps due to the presence of degradative enzymes.

Planned and ongoing work includes: 1) exploring the possibilities of metabolic interrelationships between lipoteichoic acid and lipoteichoic carrier; 2) characterization of LTA degrading enzymes; 3) testing mesosomal protein and lipid to see if it

arises from old or new membrane in pulse-labeling experiments; 4) continued attempt to determine the intracellular location of LTA in S. faecalis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00002-13 LSD

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on the Nutrition, Physiology and Ultrastructure of Staphylococcus aureus.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. S. Theodore	Res. Microbiologist	LSD, NIAID
Other:	E. Huff	Scientist Director	LSD, NIAID

COOPERATING UNITS (if any)

B. Fraser, Lab. of Immunogenetics, NIAID
J. E. Bennett, J. Kwon-Chung, Clinical Mycology Section, Lab. of Clinical Investigation, NIAID

LAB/BRANCH

Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	1	2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Gram positive bacteria contain lipoteichoic acid and have a membrane system consisting of a plasma membrane and mesosomes. The aim of this project is to correlate membrane structure with function using Staphylococcus aureus as the model test system. The major emphasis of this study is to ascertain the function (or functions) of mesosomes which contain the lipoteichoic acid present in the cell. The topics of present interest are: distribution of lipoteichoic acid during growth; isolation, purification and characterization of lipoteichoic acid; and immunochemical localization of lipoteichoic acid.

PHS-6040

Project Description

Distinct features of Gram positive bacteria are the conspicuous appearance of mesosomes (intracytoplasmic membranous organelles which arise from the membranous septum and precede crosswall formation) and lipoteichoic acid (LTA, straight chain glycerol phosphate polymer containing a phosphorylated glycolipid which acts as an acceptor molecule in the synthesis of wall teichoic acid). In S. aureus, LTA is associated with the mesosomal membrane. The overall objective of this study is to analyze the chemical composition and enzymatic activities of mesosomes and ascertain whether they are functional organelles.

1. Localization of LTA during growth. Subcellular fractions (mesosomes, membranes, periplasm, and cytoplasm) from early, mid, late log and stationary phase cells were fractionated and analyzed for LTA. Fractions were isolated by protoplasting with lysostaphin and purified by differential centrifugation. Protoplasting conditions were adjusted to a minimal time (10 minutes) to prevent any degradation and cross contamination between the various cell fractions. Each fraction was extracted with chloroform/methanol (2/1), 80% hot aqueous phenol (68 C), DNase and RNase treated, and purified on sepharose 6b columns. In each growth phase the LTA was associated with the mesosomal membrane fraction which gave one high molecular weight peak (peak I) on sepharose 6b and a glycerol to phosphate ratio of 1. No peak I material was ever detected in the periplasm, cytoplasm or culture fluids. Membrane fractions from mid, late log and stationary phase cells did contain peak I material; however, it was present to the extent of less than 10% of the total found in the cell. A second peak of lower molecular weight species (peak II) was also detected in periplasm, cytoplasm and membrane fractions, but not in the mesosomal fraction. This material also contained, to a lesser extent, glycerol with a glycerol to phosphate ratio of 0.3 to 0.5. At the present time it is not known whether this is a deacylated form of LTA or cell wall contaminant. Chemical analysis of the Peak II material is now in progress.

Isolation, purification, and characterization of LTA. To determine optimum procedures for the preparation of sufficiently pure LTA for structural analysis, a variety of extraction procedures were tested. Whole cells and cell free extracts (Braun disintegration) were treated with chloroform/methanol followed by either hot water (100 C) or 80% aqueous phenol (4 C and 68 C) extraction. The purest yield of LTA was obtained from cell free extracts treated with hot aqueous phenol followed by DNase and RNase treatment and subsequent separation by gel filtration (sepharose 6b). On a dry weight basis, the amount of LTA

obtained was equivalent to 2% of the dry weight of the cell. No protein was detectable either by 260/280 or Lowry protein. Nucleic acid content was less than 0.3%. No amino sugars were found and the only amino acid present was d-alanine as determined by the amino acid analyzer. Gas chromatographic analysis for fatty acids showed the presence of six fatty acids (C15, 16, 17, 20, 22, and 24) which comprised 5% of the total weight of the LTA. The C15 fatty acid was most prevalent and accounted for 46% of the total fatty acids. Based on per mg of LTA, there are 3.75 micromoles of glycerol and 3.51 micromoles phosphate which gives a glycerol to phosphate ratio of 1.08. Studies are now in progress on the carbohydrate composition of LTA. (In collaboration with B. Fraser, LIG, NIAID)

Immunochemical localization of LTA. Chemical localization of LTA in S. aureus is well established; however, immunochemical localization is still lacking and earlier attempts to prepare a specific antisera to LTA have failed. Using purified LTA isolated from mesosomes (80% cold aqueous phenol extraction) and a prolonged immunization schedule, we obtained antisera which gave one strong precipitin band with homologous antigen and with deacylated LTA. The IgG fraction has been obtained and is now being used to localize LTA in thin sections by immunoelectron microscopy.

Although the evidence is still indirect and pending on the outcome of the immunoelectron microscopic localization of LTA in intact cells, there is sufficient chemical data to support the localization of LTA in mesosomes. Since mesosomes are most prominent at cell division, arise from the membranous septum and precede crosswall formation, and contain LTA; it is quite reasonable to assume that they may play a vital role as active sites of LTA, cell wall and membrane biosynthesis.

The planned course of this project will include immunoelectron microscopic localization of LTA in intact cells; characterization of the LTA structure; and biosynthesis of LTA and its function as it relates to the mesosome.

2. Crytococcus neoformans metabolism. Last year we reported that C. neoformans serotypes B and C were biochemically distinct from serotypes A and D with respect to malic acid utilization (A and D types have a defective transport system) and creatinine metabolism. (All 4 types utilized creatinine but only the B and C types produced ammonia.) Now we have developed an enzyme assay for the degradation step of creatinine using cell free extracts prepared by Braun disintegration. Ammonia production

from creatinine was 15 fold greater with B and C serotypes than the A and D types. The simplicity of this assay can now be adopted as a diagnostic tool for the differentiation of C. neoformans serotypes. (In collaboratory with J. E. Bennett and J. Kwon-Chung, LCI, NIAID)

Publications

Bennett, J. E., Kwon-Chung, K. J., and Theodore, T.S.: Biochemical differences between serotypes of Cryptococcus neoformans. Sabouradia 16: 1-8, 1978.

Kwon-Chung, K. J., Bennett, J. E., and Theodore, T. S.: Cryptococcus bacillisporus sp. nov. Int. J. Syst. Bact. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00004-18 LSD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Electron Microscopy of Bacteria in Relating Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. M. Cole	Chief, LSD	LSD, NIAID
Other:	T. J. Popkin	Chemist	LSD, NIAID
	D. L. Williamson	Visiting Investigator From SUNY, Stonybrook, N. Y. (IPA)	LSD, NIAID

COOPERATING UNITS (if any)

See following page for cooperating units.

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20014

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1.3	0.3	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project provides support, through preparative techniques (negative staining, thin sectioning) and subsequent examination, for Laboratory and Collaborative microbiological studies requiring transmission electron microscopy. Objects of ultrastructural study include mycoplasmas and their viruses; streptococci and their bacteriophages; staphylococci; bacilli; gram-negative bacteria; fungi; and various cell fractions thereof, examined by means of cytochemistry and immunoelectron microscopy.

Cooperating Units:

Dept. of Cellular and Developmental Biology, University of Arizona, Tucson (N. F. Mendelson)

Laboratory of Clinical Investigations, NIAID (Clinical Mycology Section; K. J. Kwon-Chung)

Laboratory of Infectious Diseases, NIAID (Mycoplasma Section; J. G. Tully) Z01 AI 00027-11 LID

Office of the Scientific Director, NIAID (R. Repaske) Z01 AI 00132-11 OSD

Clinical Pathology Department, CC, NIH (Microbiology Service; J. D. MacLowry)

Division of Bacterial Products, BOB, FDA (C. M. Hardegree)

Plant Protection Institute, USDA, Beltsville (Bee Environmental Studies Division; T. Clark)

Plant Protection Institute, USDA, Beltsville (Entomology Division; R. F. Whitcomb)

Project Description:

Objectives are to provide, as required, electron microscopy facilities in support of other projects in the Laboratory; and to conduct such original and in depth studies of the ultrastructure of micro-organisms as may be engendered by collaborative beginnings or by independent approach to problems.

1. The morphology and ultrastructure of spiroplasmas, as well as their carriage of viruses, was continually monitored, and 10 new isolates from ticks, bees, cactus, lettuce, and flowering trees were added to the +20 strains already under surveillance. All new isolates, except one, carry viruses SVC1 or SVC3; and by use of 2 bee strains, a plaquing and propagating system for SVC1 was finally achieved. EM was employed for following stages of purification and characterization of this virus and of SVC3. (In collaboration with D. Williamson; T. Clark and R. F. Whitcomb, USDA, Beltsville; and J. G. Tully, LID, NIAID, Project No. Z01 AI 00027-11 LID; see also Project No. Z01 AI 0007-4 LSD)

2. With D. L. Williamson, we initiated studies of the periodically structured internal filaments of spiroplasmas revealed by EM after physical disruption or treatment with detergents or digitoxin. These filaments, which may be related to actin or to

bacterial elongation factor (EF-Tu) and could be responsible for spiroplasmal motility, can be produced in quantity; and will be purified and examined immunocytochemically with anti-actin and other appropriate reagents.

3. A number of strains of Acholeplasma laidlawii were examined by both negative staining and sections to follow the course of infection by viruses MVL1, MVL2, and MVL3--as well as to determine the natural carriage of any of these viruses. Several strains of mycoplasmas, including new isolates from flowers and ground squirrels, were examined for morphologies and the possible presence (unconfirmed) of viruses. (See Project Z01 AI 00027-11 LID, Dr. Tully)

4. Bacteriophages of gorup A streptococci (Project No. Z01 AI 00005-13) and group H streptococci, E. coli, and H. influenzae (Project No. Z01 AI 00006-7) were examined for morphologic confirmation, in the detection (after induction) of lysogeny, or during procedures of purification.

5. Cultures of Philadelphia strain #2 of the Legionnaires' Disease Organism (supplied by Dr. J. MacLowry, Microbiology Service, CC, NIH) were examined by negative straining and sections. The ultrastructure of this organism was not previously reported in the literature, but an erroneous and misleading article appeared after our work was completed. The rod-shaped bacterium is a simple one, without flagella or pili, and possesses a double membrane type of envelope typical of gram-negative bacteria. Intracytoplasmic granules are common. The findings were used in a collaborative study with F. C. Chandler, CDC (see publication) to confirm the nature of the organism as found in sections of lungs of confirmed human cases, and will be presented separately in a future publication.

6. Strains of Filobasidiella (formerly Cryptococcus) and some apparently related fungi were examined in thin section to confirm or deny important taxonomic criteria such as clamp connections, binucleate filaments, and dolipores. (J. Kwon-Chung)

7. Miscellaneous samples examined included cells of E. coli to determine the effect of CO_2 on cell growth and septation (R. Repaske, OSD, NIAID); elongated and helically twisted filaments of B. subtilis mutants to determine "handedness" of the helix and nature and number of filaments in each bundle (N. Mendelson, University of Arizona); and red cells and cholesterol suspensions treated with tetanolysin, to confirm the formation of crescentic cholesterol-lysin complexes characteristic of other oxygen-labile hemolysins (C. Hardigree, BOB, FDA).

Owing to the largely support function of the operation, the course of subprojects is unpredictable--but they will be continued or curtailed as required by results.

Publications:

Chandler, F. W., Blackmon, J. A., Hicklin, M. D., Cole, R. M., and Callaway, C. S.: Ultrastructure of the agent of Legionnaires' Disease in human lung. Am. J. Clin. Pathol. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00005-13 LSD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies on Bacteriophages and Genetics of Streptococci

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Colon Whitt	Microbiologist	LSD, NIAID
	R. M. Cole	Chief, LSD	LSD, NIAID

COOPERATING UNITS (if any)
Laboratory of Microbiology and Immunology, NIDR, NIH (B. Chassy)

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.1	1.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range objective of this project is to study genetic exchange in streptococcus; primarily lysogenic conversion, transduction and transformation, and to determine how the acquisition of altered or new genetic material can affect some of the immunological and pathogenic characteristics of these organisms. At present we are investigating the identity and site of the genetic coding of an erythrogenic toxin produced by a rare non-lysogenic group A strain. Current findings verify the identity of the toxin and suggest that the genetic information may be found in a deficient prophage or in an integral segment of the streptococcal genome. Future studies will attempt to elucidate the genetic mechanism for the production of the three types of erythrogenic toxins in different toxinogenic strains.

Project Description:

Erythrogenic toxin (ET) or streptococcal pyrogenic exotoxin (SPE)is an extracellular product of group A streptococci. Four ET have been described, (A, B, C, and D), and the lysogenic state has been considered essential for their production. However, the relation of lysogeny (nearly all group A strains are lysogenic) to specific toxins has not been defined. Last year we reported production of ET type B by a rare non-lysogenic strain (64 x 402). Lysogenization or vegetative infection of this strain with a phage isolated from strain C203U (producing ET A, B, and C) induced 64 x 402 to newly produce C toxin (but not A toxin) in addition to the B toxin already produced. These results suggested that, contrary to previous reports, lysogenization may not be essential for the synthesis of all ET and raised the question of the location of the genetic coding of these toxins, particularly B toxin.

Further studies have been done 1) to affirm the identity of the B toxin produced by strain 64 x 402; 2) to attempt to confer production of ET to some other streptococcal strains by vegetative infection with phages isolated from toxinogenic strains; and 3) to attempt to find the location of genetic coding of B toxin in strain 64 x 402.

The identity of the B toxin produced by strain 64 x 402 was further verified by direct comparison with an isoelectrically purified B toxin (kindly supplied by Dr. Dennis Watson, University of Minnesota). Using monospecific B antitoxin as antibody, double immunodiffusion tests showed a line of identity between the B toxin antigen control and the test antigen (ammonium sulfate concentrated supernatant of 64 x 402). Furthermore, gel electrophoresis of the control and test samples showed a common band migrating similarly, although other components were also found in the test sample.

The first studies were done with one strain, non-lysogenic 64 x 402, and one phage, AT 203U. To clarify whether toxin production can be conferred by lysogenization or vegetative infection, other strains and phage combinations were studied. Group A non-toxinogenic strains K56 (Group A), 867 (Group C), 722 (Group C) and toxinogenic strain 64 x 402 (Group A) were vegetatively infected with bacteriophages from group A toxinogenic strains NY5 (ET-A and B), C203U (ET- A, B, and C), T18 (ET-C) and T19 (ET-A and B). The filtered lysates were concentrated 100X by either using an Amicon cell or by ammonium sulfate precipitation. The concentrated fluids were tested for toxin production against monospecific antitoxins A, B, or C, by the Ouchterlony test. No evidence of any of the three toxins was found. None of the strains were lysogenized by any of the phages.

Production of B toxin by non-lysogenic strain 64 x 402 indicated that the genetic coding cannot be on an intact prophage genome but may be 1) an integral segment of the streptococcal chromosome, or 2) an integrated portion of an incomplete prophage genome, or 3) a transduced fragment, or 4) an integrated plasmid or transposon, or 5) a free plasmid or transposon.

These hypotheses were tested by transformation experiments. If the genetic information was present in extracted DNA in an adequate number of copies, there was the possibility that the ED^+ marker could be transferred to an ET cell by transformation. Streptocmycin-resistant gorup A strain NY5 Sm^R (ET-A and B) was used as DNA donor and group H strain Wicky as DNA recipient. The streptomycin marker was used as control to test DNA integration. Transformation experiments were performed by adding NY5 Sm^R DNA to competent Wicky cells. The mixture was incubated for 30 minutes, diluted 1:10 in dialysate media and incubated for 4 hours. This culture was added to 400 ml of the dialysate medium, incubated overnight, centrifuged, and concentrated by $(NH_4)_2SO_4$ precipitation. The concentrated fluid was tested for the presence of A and B toxins by Ouchterlony tests using A and B specific antitoxin. Although there was transformation of the Sm marker (showing DNA uptake and integration), there was no evidence of ET production.

No plasmids were found in strain 64 x 402 by either Dr. Bruce Chassy (LMI, NIDR) or Dr. Alan Liss (LSD), using different methods independently.

These results indicate that the toxin of strain 64 x 402, though not highly purified in preparations used for gel electrophoresis of proteins, is probably identical to the isoelectrically-purified B toxin used as a standard. The failure of phages from toxinogenic strains to stimulate production of ETs in other strains needs further explanation, but may not be totally unexpected in view of the known similar failure of some phages from tox^+ strains of Corynebacterium diphtheriae--in which toxin production is a complex interaction between host and the phage carrying the tox^+ marker. Similarly, the failure to transform ET production to a group H streptococci (which was selected because, unlike members of groups A, C, and G, it never produces such toxin naturally) may be due to factors of integration and expression that are not yet understood. Though it appears that the coding for B toxin is not on a plasmid; and in strain 64 x 402 is not related to a lysogenic state, we have not yet been able to make a determination among other possibilities.

Future work will be directed to other recipient cells, other phages, and other methods for transferring and localizing the DNA coding for B toxin.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00006-7 LSD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Competence Development and Genetic Exchange Mechanisms in Streptococci.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. M. Ranhand	Sr. Scientist	LSD, NIAID
Other:	D. Moynet	Visit.Fellow (France)	LSD, NIAID
	E. Jablonska	Visit.Fellow (Poland)	LSD, NIAID

COOPERATING UNITS (if any)
Macromolecular Biology Section, LBV (C. F. Garon) Z01 AI 00156-03 LBV
Macromolecular Biology Section, LBV (F. DeFilippes) Z01 AI 00126-05 ~~LBV~~

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INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20014

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2.5	2.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project as originally defined, "to study the mechanism by which competent group H streptococci take up deoxyribonucleic acid (DNA) molecules from their surroundings", is continued.
I examined the interaction of competent Wicky cells with homologous and heterologous DNAs and found that most DNAs that are purified by conventional methods still contain an unidentified protein. This protein allows DNA to bind to competent cells in the absence of certain divalent cations. When the protein is removed, DNA no longer binds in the absence of certain divalent cations.
In conjunction with the observation, I examined the role of divalent cations in the cell-DNA reaction and found that calcium is solely responsible for the total effect: binding, entry, and production of acid-soluble products.
The results thus far obtained further our understanding of how competent cells interact with DNA.

PHS-6040

Project Description:

The object of this work is to study the competent streptococcal cell-DNA reaction. Only competent cells are physiologically capable of interacting with DNA molecules and four interactions are defined. (1) the binding of DNA to competent cells presumably at specific surface receptor sites, (2) entry of DNA into the cells' cytoplasm, (3) the production of acid-soluble products (ASP) from the DNA (by specific nucleases?), and (4) genetic recombination and expression of homologous DNA (transformation). Entry and ASP production may be inter-related. Reactions (1) to (3) which occur during a 30 minute period at 37 C are measured by using radio-labelled DNA; (4) is measured by determining the number of cells that acquire one or more new traits.

Last year I reported that all of the above steps could be satisfied by calcium. The saturation concentration has been determined to be 0.8 mM; concentrations up to 25 mM have been used in most cases. I also reported that inhibition of reactions (2) and (3) by chelators (EGTA, EDTA) could not be restored with excess Ca, Mg, or Ba. It was of interest, therefore, to determine the cation requirement for these steps. None were found. I tried Zn, Fe, Cu, Mn, Cd, Co, and Ni.

When competent cells were washed (at 0 C) with an EGTA solution and then placed in a medium containing Ca (free of EGTA) all of the above steps were manifested. In addition, steps (1) to (3) were carried out by cells that were treated with EGTA for 30 minutes at 0 C, and then treated with Ca (free of EGTA). These results suggest that the action of chelators might require an activation energy. At 37 C, no ^{14}C-labelled EDTA (less than 0.05%) was found associated with cells. It is possible that chelators translocate or solubilize essential factors other than divalent cations.

In summary, the following results were obtained. Step (1) occurred to various degrees with either Ca, Zn, Fe, or Cu. Step (2) was stimulated only by Ca. Step (3) was stimulated to various degrees by Ca, Mn, Co, Mg, Fe., Cd, and Ni. Without step (2) step (4) cannot occur. In combination with Ca, different cations inhibited to various degrees step (2) and in some cases stimulated step (3). The stimulation of step (3) was synergistic and suggests that two or more nucleases are involved with the production of ASPs. An examination of the degradation products and/or isolation of specific mutants might support this hypothesis.

A calcium related observation also made last year was: homologous DNA was capable of binding to competent cells (step 1) in the

absence of added divalent cations (Ca) whereas heterologous T7 bacteriophage DNA was not. In the presence of Ca, both DNAs were carried at least through steps (1) to (3) above.

To study this phenomenon, I purified DNAs from various bacteriophages and bacterial cells and can now partially explain the observation. Homologous and heterologous DNAs contain, after "purification" by standard methods, a protein that allows the DNA to bind to competent cells in the presence or absence of cations. Some bacterial virus DNAs (T7, T3, A25) are apparently free (or freed) of this protein or they contain lesser amounts.

The protein can be removed (or inactivated) from the DNA by various treatments: (a) proteolytic enzymes, (b) precipitation with ethanol, and (c) heating at 70 C for 60 minutes in 0.1 M salt. For unknown reasons, any one treatment is not applicable to all DNAs. When the protein is removed (or inactivated) competent cells do not undergo step (1) unless a divalent cation is supplied.

I am continuing studies on the cell-DNA interaction and will try to localize the protein on the DNA molecule. I will also continue studies on the physiology and enzymology of competence.

Dr. Jablonska examined some properties of phage N3 grown in Haemophilus influenzae Rd9(N3). Phage DNA was extracted and examined by velocity and equilibrium centrifugation, restriction enzyme analysis using agarose gel electrophoresis, electron microscopy, thermal melting curves, and transfection experiments after the DNA had been treated with various agents. This double stranded DNA, molecular weight 26×10^6 daltons, has continuous single strands at 13×10^6 daltons as shown by alkaline sucrose gradient centrifugation using both lambda and T7 DNA as markers. The duplex form, however, readily aggregated in neutral salt solutions at over moderate ionic strength. Monodisperse peaks at 26×10^6 daltons were seen on neutral sucrose gradients containing 10 mM KCl, 1 mM sodium EDTA, and 10 mM Tris-HCl buffer, pH 8.3. (High salt aggregation is seen with other Haemophilus phage DNA such as from phage P HP1Cl.) Aggregation may be due to N3 DNA having "sticky" ends similar to those found in lambda DNA, although simple heating at 75 C will not linearize N3 DNA.

EcoR1, Xbal, and Xhol restriction enzymes were used to cleave N3 DNA into a relatively small number of segments (less than 10). EcoR1 digests show the presence of a large segment of DNA which can be converted to two smaller segments whose combined mass equals that of the larger segment, thus demonstrating the presence

of sticky ends. The 6 segments produced by EcoRl digestion have molecular weights of 7.05, 5.67, 4.48, 4.30, 2.71, and 2.16 x 10^6 daltons, with the 2 largest segments forming the end fragments. A circular EcoRl may was constructed from these data. CsCl equilibrium centrifugation measurements and thermal melting curves indicate a content of G + C of 43 mol. %. The fact that this DNA has a structure similar to lambda DNA but with "sticky" ends that do not behave in exactly the same manner makes it an interesting subject for further investigation. The ability of the DNA to transfect H. influenzae makes it a useful tool for correlating the biological activity of the DNA with its structure. (In collaboration with F. DeFilippes and C. F. Garon, LBV, NIAID)

Dr. Moynet has compared 4 phages isolated from saliva with a temperate phage induced previously from a group H streptococcus. All 5 phages can lysogenize, and thus inhibit transformation in, group H streptococcus WE4. All are identical morphologically (polyhedra with long non-contractile tails); have the same buoyant density (1.5 g/cm^3 in CsCl); show the same 5 structural proteins by gel electrophoresis; and contain double-stranded linear DNA of molecular weight 23.2 to 24.6 x 10^6 daltons with a G + C of 42 to 44 molecular percent by melting point determination. The DNAs are cut identically by restriction endonucleases Hind III and XBaI, but not at all by 3 others. Denaturation and annealing, including the use of Exonuclease III, show the presence of permutated and repeated nucleotide sequences and, therefore, a probable process of packaging DNA into the phage head that involves linear concatemers and excision mechanisms like those of coliphages T3 and T7. (In collaboration with C. F. Garon and F. DeFilippes, LBV, NIAID)

Publications:

Ranhand, J. M.: Growth of T-odd, T-even and Lambda bacteriophages in static cultures of Escherichia coli. In Dean, D.H. (ed.): Microbial Genetics Bulletin, Vol. 44, Ohio, Ohio State University, 1978, pp. 1-2.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00007-4 LSD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Viruses of Spiroplasmas and Mycoplasmas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.3	1.8	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is the characterization of viruses of mycoplasmas and spiroplasmas. In the latter, new studies including new isolates from ticks, bees, and flowers as well as old strains from citrus, corn, and Drosophila, confirmed by both electron microscopy and cross-plaquing that nearly all strains carry one or more of 3 morphologically distinct viruses resembling bacteriophages. New data on the polyhedral SVC3 indicated host-range differences among virus strains from different sources, showed an adsorption constant of 1.3×10^{-9} cm^3/min, a latent period of 2 hours, a burst size of 26 to 30 plaque forming units, and a K value of homologous antiserum of 880. The double-stranded DNA (MW 13.36×10^6 daltons) was found to be circularly permuted and terminally repetitious, with a GC content of 27 mol. %; it was cut by 3 restriction endonucleases (EcoR1, Hind III, Hae III) but not be a fourth (BamI). The rod-shaped virus, SVC1, was isolated from, and propagated on, a bee spiroplasma: it appears to contain DNA, and further characterization is in progress.

Project Description:

Objectives were to continue investigations of spiroplasma viruses in respect to distribution, host range, growth, and biophysical characterization.

Spiroplasma strains currently available include isolates from citrus "stubborn" disease (S. citri), corn stunt disease (CSO), ticks (SMCA, 244F and TP2), bees (BC3, KC3), cactus, lettuce, and flowers (OGMB), as well as the uncultured spiroplasma (SRO) carried in, and causing an imbalance of sex (no male progeny) in Drosophila. By electron microscopy, 93% of 56 total strains carry one or more of the 3 virus types previously described. By reciprocal cross-spotting of plate washes on lawns, 100% of 29 of the same strains form plaques on one or more lawns. Although the identity of the virus producing a plaque has not been determined in all instances, it is clear that carriaige of viruses by spiroplasmas, of whatever source, is extremely common if not universal.

In such plaquing tests, the number of plaque-forming units (pfu) produced by any one plate wash or culture supernate is consistent on any one host lawn (on repeated tests)--but differs from one lawn to another. In addition, the 2 viruses (SVC3/608; SVC3/SMCA) previously purified from different sources show differences in host range. Both these results suggest the presence of immune or host restriction and modification systems that will require further study which is under way. The situation is complicated by the finding that several strains appear to be self-plaquing or self-inducible when lawns are spotted with homologous washes.

Characterization of the polyhedral short-tailed virus, SVC3/608, was continued. New data include an adsorption rate of 1.3×10^{-9} cm^3/min; a latent period of 21 hours; a burst size of 26-30 pfu; and a K value of 880 for homologous antiserum. Further physicochemical studies of the viral DNA, shown previously to be double-stranded and linear with a molecular weight of 14×10^6 daltons, show that it has a G + C content of 27% by either thermal denaturation or isopycnic banding in cesium chloride; and, as shown by denaturation or Exonuclease III treatment followed by annealing, exists as a series of strands with both a permutated arrangement and repetition of nucleotide sequences. The DNA is cut by restriction endonucleases EcoR1, Hind III, and Hae III, but not by Bam I. Planned studies include comparisons of above findings with similar absorption, one-step growth, serologic tests, and DNA characterization for SVC3/SMCA--which has now been cloned.

A second virus, the rod-shaped SVC1, was at last plaqued and propagated. Success was achieved with 2 bee spiroplasmas--strain KC3 as donor and BC3 as propagating strain. The virus bands at 1.39 g/cm^3 in cesium chloride gradients, and 1.21 in metrizamide. Its host range appears somewhat restricted, since it plaques on only 21% of 25 spiroplasma lawns tested. Preliminary tests with diphenylamine (positive) and orcinol (negative), and uptake of radiolabeled thymidine (positive) and uridine (negative) suggest that the viral nucleic acid is DNA. Current studies are directed to defining adsorption, one-step growth, structural proteins of the virion, and the strandedness, molecular weight, and sequence arrangement of the DNA. This virus provides a second probe (in addition to SVC3) for investigating basic aspects of infection and replication in wall-less prokaryotes, and for approaching problems of evolution, taxonomy, and pathogenicity in an increasing number of unique mycoplasmas.

Publications:

Cole, R. M., Mitchell, W. O., and Garon, D. F.: Spiroplasmavirus citri 3: Propagation, purification, proteins, and nucleic acid. Science 198: 1262-1263, 1977.

Cole, R. M.: Mycoplasma and spiroplasma viruses ultrastructure. In M. F. Barile and S. Razin (eds.) The Mycoplasmas, Volume I, Cell Biology. Academic Press, N. Y. (In press)

LABORATORY OF BIOLOGY OF VIRUSES
1978 Annual Report
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Annual Report of the Laboratory of Biology of Viruses
National Institute of Allergy and Infectious Diseases, NIH
October 1, 1977 to September 30, 1978

The goal of the Laboratory of Biology of Viruses is to determine how viruses replicate. At the molecular level, this involves investigation of the structure of the virion and of its purified component parts, of mechanisms of replication and transcription and expression of the viral genome. Emphasis is also placed on cell-virus interactions including the process of cell transformation. We believe that information of this kind, although difficult to obtain, is fundamental to understanding disease processes and vital to achieving a rational basis for chemotherapy.

PAPOVAVIRUSES

SV40 DNA Synthesis.

In previous studies, we have characterized intermediates in SV40 DNA replication by using DNA extraction conditions which disassociate protein from nucleoprotein complexes. In our current studies, we have extracted cells under conditions where the integrity of viral nucleoprotein complexes is maintained. These complexes can support the continued replication of SV40 DNA *in vitro*. Both cellular and viral chromatin can exist in at least two distinct configurations. In one form nucleosomes appear as beads on a string in a 100-120 Å fiber. Alternatively, chromatin can exist as 200-300 Å fibers. We find that replicating DNA nucleoprotein complexes exist as 100-200 Å fibers with nucleosomes present on both sides of the replicating fork; and that progeny chromosomes also have this same structure. This structure is not sufficiently compact to be incorporated into virions. This implies that after completion of DNA replication, the structure must undergo additional folding prior to virion formation.

Using these replicating nucleoprotein complexes, we have re-examined why replication of progeny SV40 DNA proceeds more slowly than predicted based on the rate of chain elongation. We find that in these complexes the molecules grow to full length, but there is a block when these almost completed molecules have to undergo separation to give rise to two new daughter molecules. (Seidman, Salzman)

Transcription and Processing of Viral RNA Transcriptional Intermediates (TI's)

The levels of TI's and the specificity of the regions of the genome that are transcribed by TI's, when compared with the appearance of m-RNA species in the cytoplasm, provides good evidence that TI's are responsible for RNA synthesis *in vivo*. However, there may be a clear difference between *in vivo* and *in vitro* results when the properties of TI's are examined under conditions where only early m-RNA has been found *in vivo* in either the nucleus or the cytoplasm. These conditions are either at early times in a lytic cycle prior to initiation of viral DNA replication

or under conditions where cells have been infected with a tsA mutant at a restrictive temperature in order to block DNA synthesis. We have examined the in vitro pattern of synthesis when cells are infected with tsA58 SV40 at a restrictive temperature and TI's are prepared 12-14 hr post infection. Under in vitro conditions the majority of RNA species (60-70%) are made from the late strand, although RNA transcribed from the early region of the early strand is also readily seen.

Based on our data with transcriptional intermediates, we have proposed a model to explain temporal regulation of transcription. We propose that input viral DNA has two promotors and both are available. They give rise to early and late transcripts. Early transcripts code for T antigen and it suppresses the synthesis of additional T antigen. We are currently testing different predictions based on this model (Chiu, Birkenmeier and Salzman).

Studies of Viral RNA under In Vivo Conditions.

By pretreating SV40 infected BSC-1 cells with glucosamine, ^{3}H-uridine labeling of both cellular and viral RNA can be halted instantaneously by addition of cold uridine. We have studied the fate of pulse-labeled viral RNA from cells at 45 hr postinfection under these conditions.

During a 5 min period of labeling, both the messenger and non-messenger regions of the late strand are transcribed. After various chase periods, nuclear viral species which sediment at 19, 17.5 and 16S are observed. Of the material present at the beginning of the chase period, 50% is degraded rapidly with a half life of 8 min (initial processing). This rapidly degraded material is that fraction of the late strand which does not give rise to stable late m-RNA species. Forty percent is transported to the cytoplasm and 10% remains in the nucleus as material which sediments in the 2-4S region. These 2-4S viral RNAs have a half life of 3 hr and hybridization studies suggest that they are in part coded for by the late strand non-messenger region and are derived from the initial nuclear processing step.

Transport of nuclear viral RNA into the cytoplasm is detected after a 5 min pulse and a 7 min chase. The maximum amount of labeled viral RNA is accumulated in the cytoplasm after a 30 min to 1 hr chase (Chiu, Salzman).

Mechanisms of RNA Splicing

During the past two years, a series of papers have described the generation of species of m-RNA molecules which arise from two distinct and separate DNA sequences by splicing. In the case of SV40, the integrity of the coding sequence is preserved and a leader sequence is spliced to it. This mechanism of splicing is being examined by a technique which first involves the isolation of specific regions of the viral genome that are generated by restriction enzyme cleavage and are fractionated by column chromatography. These fragments are then annealed to viral

m-RNA and the DNA is used as a primer for the enzyme, reverse transcriptase, and a complementary DNA copy of the m-RNA is made. This DNA is then sequenced. The sequence of the c-DNA has been compared with the known nucleotide sequence of SV40. The regions of the DNA that have been deleted, and the nucleotide sequence of the splice have been exactly determined. (Bina-Stein, Thompson, Salzman)

Structure of Early m-RNA Species.

Using column chromatography and restriction enzyme cleavage of SV40 DNA, radiolabeled probes of 20 to 200 nucleotides which correspond to known regions of the viral genome have been isolated. These have been hybridized to early viral m-RNA and the reaction products fractionated by velocity sedimentation. This procedure has provided direct physical evidence for two early m-RNA species that contain different spliced structures (Thompson, Bina-Stein, Salzman).

Processing of Nuclear RNA.

A fraction of nuclear RNA is known to be resistant to RNA'ase digestion. These regions presumably consist of self complementary regions and they may serve as signals in RNA processing. Since these regions are double stranded, we have used compounds which interact with double stranded nucleic acid to see how they affect RNA metabolism. We find that Proflavin blocks RNA processing, and thus its transport from the nucleus. We are currently studying how synthesis of specific RNA sequences are affected by this drug (Chiu, Bruszewski, Salzman).

PARVOVIRUSES

Adenovirus-associated Virus (AAV).

Virus Protein Plays Role in DNA Replication.

To study further the mechanism of viral DNA replication in vivo, AAV DNA synthesis was analyzed under conditions which restrict virus-specific protein synthesis. It was found that AAV structural protein is required for the efficient processing of AAV DNA concatemers to unit length molecules. In the presence of a protein synthesis inhibitor, cyclohexamide, or in primary monkey kidney cells in which AAV messenger RNA is not translated, high molecular weight concatemers accumulate, and single-stranded progeny molecules are not generated. These findings suggest that AAV structural protein plays more than one role in the viral replication process: (i) it may be required for specific processing of concatemers (itself an endonuclease) or, coupled with a cellular endonuclease, may simply enhance the efficiency of processing by a cellular enzyme, (ii) it may be required to prevent reinitiation of DNA synthesis on single strands displaced from replicating DNA (i.e., prevention of conversion of single strands to double-stranded replicating intermediates) and (iii) it serves to encapsidate separate plus and minus progeny strands. Because the AAV DNA replication process probably shares much in common with that of the cell, definition of specific

requirements for AAV DNA synthesis should provide insight into the mechanism of cellular DNA synthesis. We plan to continue to study these requirements with this latter objective in mind (Sebring, Buller and Rose).

Human Cell Endonucleases are Characterized.

The enzyme(s) responsible for AAV concatemeric processing may also play a role in normal cellular DNA synthesis. We have now purified two single-strand-specific endonucleases from KB cells as possible candidates for such processing enzymes. The first enzyme has a pH optimum at 9.2, requires Mg^{2+} for activity, and is inhibited by mono- or divalent cations. Its sedimentation coefficient of 4.6S is based on sucrose gradient sedimentation, and it has a molecular weight of 54,000 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme specifically catalyzes the endonucleolytic cleavage of denatured DNA, yielding acid-soluble oligonucleotides which contain 5'-phosphoryl termini. The rate of hydrolysis of poly(dT) is approximately eight-fold greater than that observed with denatured DNA, although the K_m for both substrates is 1.74×10^{-5} M. The relative rates of hydrolysis of homopolymers by the endonuclease are poly(dG)>poly(dT)>poly(dA)>poly>(dC). Purified enzyme preparations also hydrolyze poly(U), releasing acid-soluble products. This activity cosediments in sucrose gradients with the DNA endonuclease activity, suggesting that both activities are contained in the same enzyme molecule. The second enzyme has a pH optimum at 7.4 and hydrolyzes poly(dT) relatively faster than the other homopolymers. This enzyme appears to have a high degree of sequence specificity. Efforts will be continued to attempt to identify and characterize enzymes involved in virus and cellular DNA synthesis (Wang and Rose).

Herpesvirus Now Shown to be AAV Helper.

To investigate the basis of the AAV multiplication block in herpesvirus (HSV)/AAV-infected cells, quantitative and kinetic parameters of AAV DNA, RNA and protein synthesis were compared in HSV/AAV and adenovirus(Ad)/AAV-infected KB cells. Several distinct differences were found. In HSV/AAV infections the intervals between virus addition and detectable initiations of AAV-specific DNA, RNA and protein synthesis were substantially diminished, and the maximal rate of synthesis in each case was maintained for a shorter period of time. Also, AAV RNA synthesis commenced simultaneously with AAV DNA synthesis in contrast to the delayed onset of AAV RNA synthesis in Ad/AAV infection. This RNA, however, exhibited a shorter half-life in the cytoplasm of KSV/AAV-infected cells. Finally, electron microscopic and hybridization studies detected significant quantities of progeny AAV particles in isopycnic gradient analyses of HSV/AAV-infected cultures although approximately 1000-fold lower than in comparable Ad/AAV infections. These data indicate, for the first time, that HSV is capable of acting as a complete helper for AAV multiplication, but that this capability is relatively inefficient. Thus, herpesviruses appear to possess the ability to perpetuate naturally defective parvoviruses.

This may explain, in part, the latent persistence of AAV in certain cells, and is a fact that should be considered in the preparation of some vaccines (Buller, Sebring and Rose).

Adenovirus Helper Factor(s) is not Expressed in Transformed Cells.

To test whether Ad functions expressed in malignantly transformed cells were capable of inducing AAV replication, AAV infection of Ad-transformed human kidney cells was investigated. The transformed cell line synthesized T antigen(s) as well as 72K DNA-binding protein. Synthesis of AAV nucleic acid could not be detected in the absence of added helper virus (Ad 5). Therefore, in this cell line (as well as in a rodent line we previously tested) the Ad requirement(s) for AAV replication was not synthesized. Thus, Ad DNA-binding protein and those proteins mapping within the Ad transforming sequences (first 5-10% of the left end of the genome) do not assist AAV replication (at least without some other adenovirus protein not expressed in the transformed cells). We plan to continue studies directed at identifying and isolating the specific AAV helper factor(s) supplied by both adenoviruses and herpesviruses. Results from our previous studies indicate that this factor(s) may be involved in initiation of AAV DNA synthesis. Confirming and understanding its role in initiation of AAV DNA synthesis should provide insight into the mechanism of initiation of adenovirus and herpesvirus DNA synthesis. This kind of information is relevant to possible selective interference with virus infection (Bloom and Rose).

Kilham Rat Virus (KRV).

The parvovirus, KRV, is able to replicate autonomously in rapidly dividing cells. The virion contains three proteins and one molecule of linear single-stranded DNA with a molecular weight of about 1.6×10^6. During the last year we determined that the linear single-stranded viral DNA contains termini on both the 3' and 5' ends which are resistant to the single-stranded specific endonuclease S1. These termini composed of 90 to 150 nucleotides are believed to have a hairpin configuration. (L. Salzman) The hairpin at the 3' end of the molecule can serve as a self-primer for synthesis of a double-stranded linear intermediate. We have utilized the ability of the viral KRV-DNA to self-prime in the presence of the "large fragment" of E. coli DNA polymerase to synthesize a completely double-stranded molecule. We have cleaved the viral DNA at intervals during the synthesis of the duplex molecule and hydrolyzed it with restriction endonucleases. Using this procedure, we obtained restriction enzyme fragments in the order of their synthesis and have prepared a linear map of the fragments. (L. Salzman, Wali and Garon). This in vitro synthesis of pure preparations of double-stranded DNA using virion DNA and DNA polymerase and the knowledge of the sequence of restriction enzyme fragments will aid us in further studies. We hope to sequence the nucleotides at the 3' terminus and the adjacent area to gain information about the initiation sequence for DNA polymerase. (L. Salzman). From use of restriction enzyme fragments we may be able to detect and sequence the attachment site for RNA polymerase and for the mRNA.

A double-stranded DNA intermediate has been implicated in KRV-DNA replication but it is difficult to study or purify the small in vivo synthesized KRV-DNA intermediate molecules. We hope to learn more about DNA synthesis and the replicative intermediates using nuclear DNA-protein complexes capable of synthesizing DNA. Such complexes have been used to study the DNA replication of adenovirus and SV40 virus (L. Salzman).

We have also recently studied the synthesis of viral specific RNA in the cytoplasm, nucleus and the polysomes of KRV infected cells. We have found by DNA hybridization, two viral specific RNA species sedimenting at 22 and 12's in aqueous sucrose gradients. In the nucleus we have also seen a 26S viral RNA which would represent an entire transcript of the viral genome. We intend to investigate the possibility of post-transcriptional processing of the 26S to the functional 12 and 22S RNA's. We also wish to determine the genome location of the viral RNA's by use of in vitro restriction enzyme fragments and visualization of "R-loops" in the electron microscope. We also intend to determine the proteins coded for by the viral m-RNA's using a reticulocyte lysate technique (Wali, L. Salzman).

In conjunction with Dr. Darrell Campbell (NCI), we have found that rat adenocarcinoma cells 13762 (a DMBA induced tumor) are strongly inhibitory to lymphocyte proliferative responses. This inhibition was due to a contamination of the line with KRV. The virus can replicate in rat lymphocytes, predominantly in lymphocytes stimulated to active proliferation. Other transplantable rat tumor lines (C58NT) D is also contaminated with KRV. The presence of such viruses in in vivo passaged tumor lines may adversely affect the measurement of lympho-proliferative responses to tumors in vitro (L. Salzman, Campbell).

ADENOVIRUSES

Inverted Repetition May Amplify Oncogenic Potency.

The structure of Ad 18 DNA from two plaque isolates derived from the prototype strain has been analyzed by cleavage with several restriction endonucleases and by electron microscopic heteroduplexing techniques. Fragment sizes were determined by contour length measurements, sucrose gradient sedimentation and agarose gel electrophoresis. A physical ordering of fragments was obtained by comparative digestion of isolated fragments and heteroduplex mapping. End fragments were identified by their susceptibility to exonuclease digestion and by the presence of covalently attached terminal proteins that remain when viral DNA is released from virions in sucrose-guanidinium gradients. It was found that plaque 2 DNA molecules were about 4% longer than those of plaque 1. In addition, the inverted terminal repetition of plaque 2 DNA was 3-4% of genome length based on measurements of duplex "panhandles" generated by self-annealing of single DNA strands. A visible "panhandle" could not be detected after self-annealing of single-stranded plaque 1 DNA. Furthermore, restriction enzyme cleavage patterns of the two

plaque isolates appeared to differ only in the size of the terminal fragments. Although these findings indicate that the greater length of plaque 2 DNA results from a longer inverted repetition, plaque 1:2 heteroduplex molecules suggest that an additional structural difference might also exist at the opposite molecular end. In contrast to previous reports of Ad 18 isolates in which enlargement of terminal sequences appear to occur in place of internal DNA sequences, these data provide evidence for a new arrangement in which sequences are added to the termini of DNA molecules with no other apparent internal molecular deletion or rearrangement. Of special interest is whether this Ad 18 genome contains a repetition of oncogenic sequences within the terminal insertion. If so, a "double dose" of transforming genes would be present and could account for the high oncogenic potency of this virus (Garon and Rose).

Noncovalent Protein Required for Formation of Circular DNA Molecules.

Robinson et al. (Virology 56, 54) originally reported that when adenovirus DNA was extracted from virions with guanidinium chloride, circular molecules could be detected by electron microscopy. It was proposed that these structures were formed as a result of a protein bound tightly to the two ends of the DNA molecule. Subsequent studies demonstrated the presence of a covalently attached, 55,000 M.W. protein. These terminal protein-protein interactions could be inhibited by treatment with SDS or a protease, resulting in linear, monomeric DNA. Brown et al. (J. Virol. 16, 366) first demonstrated that these DNA-protein complexes did not migrate into agarose gels during electrophoresis. This observation provided the basis for a useful means for detecting not only the presence or absence of terminal protein components but the identification and purification of terminal DNA sequences as well. We have fractionated the Ad 2 DNA-protein complex on hydroxyapatite columns and analyzed the DNA eluate by agarose gel electorphoresis. Although the initial DNA-protein complexed did not migrate into agarose gels, > 75% of DNA molecules in the DNA eluate were now able to enter the gels in spite of the absence of protease or SDS treatment. When the protein in the DNA eluate was labeled *in vitro* with ^{125}I, protein was not seen associated with the DNA that migrated into the gels. When the DNA eluate was examined in the electron microscope, DNA was found to be essentially linear. The addition of a DNA-free protein fraction to either the DNA eluate or to phenol extracted DNA produced circularization of about 20% of these linear molecules. We conclude that circularization of the adenovirus DNA-protein complex as well as its inability to migrate into agarose gels is likely due to an aggregate of a virion component(s) in addition to the covalently-attached 55K protein previously described (Garon and Rose).

DNA Cross-linking Reveals Sites of Interaction with Protein.

Recently a category of chemical compounds (psoralen and its derivatives) has generated considerable interest by its ability to penetrate intact cells and viruses without detectable structural alterations, to intercalate into the double helix of nucleic acid molecules and upon photochemical activation form covalent cross-links. The reaction is highly specific.

The interstrand bonds are exceptionally stable. And, unlike other cross-linking procedures, it introduces little or no damage to the DNA molecules involved. We have attempted to apply this reagent to purified adenovirus particles in search of proteins within the virion which, by virtue of their intimate association with the DNA, might interrupt the cross-linking function of the drug and thus be detectable in the electron microscope as site-specific denaturable regions. Preliminary data would indicate: (1) the effectiveness of the drug at cross-linking deproteinized, adenovirus DNA in vitro; (2) the possible identification of at least two major protein interaction sites along the adenovirus serotype 2 DNA molecule; and (3) the usefulness of the drug as a probe for freezing the secondary structure of the DNA molecules both in vivo and in vitro under various conditions of temperature and ionic strength. These sequences may represent important control elements in the expression or packaging of the adenovirus genome (Garon).

POXVIRUSES

There are several features of poxviruses that make them particularly attractive for transcriptional studies. These include (1) presence within the purified virion of all enzymes necessary for synthesis and processing of mRNA; (2) cytoplasmic site of transcription; (3) effective shut off of host protein synthesis; and (4) distinctive classes of early and late mRNAs. Continued studies with vaccinia virus, the prototype of this group, have provided the following new information.

Post-transcriptional Modification of RNA.

Enzymes that Modify the 5'-end of Viral and Cellular mRNAs.

Viral and cellular mRNAs were shown in previous years to contain a 5'-terminal cap structure [$m^7G(5')pppN^m$] consisting of a 7-methylguanosine (m^7G) linked by a 5' to 5' triphosphate bridge to one or two consecutive 2'-O-methylated ribonucleosides (N^m). During the past year, a third vaccinia virus enzyme, an S-adenosylmethionine:mRNA (nucleoside - 2'-)-methyltransferase, involved in the formation of the cap has been extensively characterized. The enzyme specifically methylates the penultimate nucleoside of RNA ending in $m^7G(5')pppN$-. This specificity indicates that 2'-O-methylation follows guanylylation and guanine-7-methylation (Barbosa and Moss).

An enzyme isolated from Hela cells, termed an S-adenosylmethionine: mRNA (2'-O-methyladenosine-N^6-)-methyltransferase, has been extensively characterized. This cap-specific enzyme methylates the $m^7G(5')pppA^m$ end of mRNA to form $m^7G(5')ppp^6m^mA$. (Keith and Moss)

The above enzymes were used to modify methyl-deficient mRNA in an effort to determine the possible roles of 2'-O-methylation and adenine-N^6-methylation of caps on translation of mRNA. We found that neither of the 2 methylations significantly enhanced ribosome binding in an in vitro cell-free system. (Keith, Cooper and Moss)

Capping and methylating enzymes from vaccinia virus were used to specifically label the 5'-ends of the genome RNAs of influenza A and B viruses. All 8 segments were found to end in pppApGpUp suggesting a special role for this conserved sequence. This novel labeling procedure should be generally applicable for sequencing the 5'-end of RNA. (Moss and Keith)

Enzymes that modify transfer RNA. A transfer RNA (cytosine-5-) methyltransferase that specifically catalyzes the transfer of a methyl group from S-adenosylmethionine to the 5'-position of cytosine residues was purified from Hela cells. A striking feature of the enzyme is its specificity for the transition region between the extra arm and right hand loop of several different tRNAs. (Keith and Moss)

Transcription of Vaccinia Virus DNA.

Multiple roles for ATP in the synthesis and processing of mRNA by vaccinia virus. In previous years we isolated several enzymes from vaccinia virus cores including two distinct ribonucleoside triphosphatates and a protein kinase, that have unknown biological functions. The utilization of ATP, however, is a common property of these enzymes. Adenosine (β, γ-imido) triphosphate (AMP-PNP), a compound that closely resembles ATP, but which has a non-hydrolyzable γ phosphate, was used to detect additional roles for ATP in transcription. We found that the analog blocks transcription at four points: (1) initiation of RNA synthesis by virus cores; (ii) chain termination and/or cleavage of nascent RNA; (iii) polyadenylylation of the 3'-end of RNA, and (iv) extrusion of RNA from the core. These findings suggest previously unknown roles for ATP in vaccinia virus transcription. (Gershowitz and Moss)

Symmetrical transcription of vaccinia virus DNA. At 5 to 7 hours after vaccinia virus infection, approximately 18% of the polyadenylylated RNA can form RNA:RNA hybrids. This RNA has a sequence complexity of about 23% of the genome and contains sequences complementary to early RNA. The possible role of symmetrical transcription in this system is under investigation. (Boone and Moss)

Inverted terminal repetition in vaccinia virus DNA. An inverted terminal repetition was observed in DNA molecules extracted from vaccinia virus. The repeated sequence was visualized by (1) nicking the hairpin loops present at the ends of vaccinia virus DNA, (ii) separating the strands of DNA by alkali denaturation, (iii) allowing the single strands to self-anneal, and (iv) examining the DNA with an electron microscope. Single-stranded circular molecules, each of which contained a duplex projection (3.54 $\pm$0.12 μm) representing the terminal repetition, readily formed. Similar size projections were also seen in heteroduplex structures formed by cross-hybridization of the separated strands of the 2 terminal Hind III restriction fragments. Based on contour length measurements and the electrophoretic mobility of the isolated inverted terminal repetition, a molecular weight of approximately 6.9×10^6 equivalent to about 10,500 nucleotides was estimated. Evidence was obtained from

DNA:RNA hybridization studies that the terminal repetition is transcribed. Preparation of more detailed transcription maps is in progress. (Garon, Barbosa and Moss)

Translation of vaccinia virus mRNA. Accurate translation of vaccinia virus is required for gene mapping studies. Previously we found that translation can be coupled to transcription in a reticulocyte cell-free system. High resolution polyacrylamide gel electrophoresis showed that at least 23 proteins synthesized *in vitro* have mobilities similar to "early" proteins made *in vivo*. Seven representative polypeptides made *in vivo* and *in vitro* were shown to be identical by partial proteolysis. Preliminary experiments also indicate that the reticulocyte cell-free system accurately translates early and late mRNA isolated from infected cells. (Cooper and Moss)

Vaccinia DNA--The Structure of the Genome and of the Covalently Linked Ends.

Vaccinia DNA has covalently-linked-complementary (CLC) ends which define each end of the physical map of the genome. Segments of the DNA produced by restriction nuclease digestion or mechanical shearing can be separated into CLC ends or interior segments by BND-cellulose chromatography after the segments have been alkali denatured and neutralized since this leaves only the CLC segments as duplex chains.

Restriction enzymes such as Hind III, Sal I, Xho I, Kpn I, Bgl II and Pvu II are used to digest vaccinia DNA to obtain unique DNA segments which are separated by agarose electrophoresis. To obtain a physical map, segments produced by one enzyme are isolated from agarose and digested by a second enzyme to find overlapping segments. End segments, such as the 2nd and 3rd largest DNA segments produced by Hind III digestion (called h2 and h3) are identified as described. Since the Hind III ends have MWs of 1.7×10^7 and 1.3×10^7 daltons they can also be used to identify end segments resulting from digestion with other enzymes by hybridization experiments.

By shearing experiments CLC fragments which are 1/4 or 1/8 etc. of the original genome length can be obtained. These long fragments are then digested with a restriction nuclease to identify groups of segments which are at each end of the molecule.

A third type of experiment generates a number of reproducible partial DNA segments each of which includes several limit product segments. This results from including drugs, such as actinomycin D and distamycin, which block certain cleavage sites, in the restriction nuclease digestion reaction.

All these approaches have been used to generate partial cleavage maps of the vaccinia genome (DeFilippes).

Studies with Molluscum Contagiosum Virus (MCV).

Molluscum contagiosum virus (MCV) is a member of the mammalian poxvirus subgroup which causes a benign tumor of the skin in humans. The disease is most common in children. Cases appear to occur world-wide and are often associated with poverty, overcrowding and poor hygiene. Molluscum contagiosum virions were isolated from clinically typical skin lesions and the DNA extracted and characterized using techniques of electron microscopy and agarose gel electrophoresis. In some experiments, a comparison of the structural features of the MCV genome and other members of the poxvirus group was made.

The genome of Molluscum contagiosum virus appears in an electron microscope as a linear, duplex DNA molecule having a mean contour length of 53.02 1.87 μm. Vaccinia virus DNA, similarly processed and used for comparison, measures 53.03 ± 2.2 μm. The average molecular weight of MCV-DNA was calculated to be 118 x 10^6 and appeared to be extremely sensitive to both mechanical shear and nuclease damage. Single-stranded circles measuring twice the length of linear molecules were observed with both poxvirus DNA's following high levels of denaturation. However, partial denaturation profiles of MCV and vaccinia virus DNA differed markedly when mounted for microscopy under similar conditions. MCV-DNA consistently required an additional 10°C equivalents to achieve comparable levels of denaturation. Of special interest was the fact that the most easily denaturable regions of the MCV genome appeared to be the ends of the molecule.

When Molluscum contagiosum virus DNA was cleaved by restriction endonuclease BAM$_6$HI, 12 fragments were produced ranging in size from 4 x 10^5 to 31 x 10^6 daltons as determined by agarose gel electrophoresis and contour length measurements. Vaccinia virus DNA, similarly treated, produced more than 23 fragments which did not appear to correspond in size to those of MCV. With the aim of comparing the genetic and structural heterogeneity of the MCV genome, eleven independently isolated virus samples were analyzed. Only 3 cleavage profiles were detected with some fragments, present in all three profiles, comigrating in agarose gels. Although we have not yet collected a large enough body of data to make an reasonable assessment of the relationship between the clinical characteristics of the disease and the restriction endonuclease cleavage patterns, it may be possible once the data are accumulated, to relate these observations and to develop a useful molecular epidemiological scheme. Such a scheme would make possible rapid identification and classification of MCV isolates without the necessity of propagating the virus in the laboratory. Furthermore, since the degree of sensitivity of such a system is limited only by the number of cleavage sites recognized by a given restriction enzyme, other available enzymes may easily be substituted.

We propose to continue to define structural features of these poxvirus DNA molecules with the aim of (1) comparing genetic variation among members of this group, and of (2) relating these features to those biochemical events which are involved in the replication of these agents. (Garon)

Administrative Changes

Our Institute's commitment and involvement in the new area of DNA recombination studies has resulted in the creation of a new laboratory which will be housed at the Frederick Cancer Research Center. Dr. Martin, the head of the Section of Physical Biochemistry, and other members of this section have been involved in recombinant DNA studies and so this group has been administratively transferred from this laboratory and into this new program area. This section will continue to be housed in the Laboratory of Biology of Viruses, but it is expected that within a year they will move to new facilities at Frederick, Maryland. Dr. Martin joined this laboratory in 1964 and in the future we will miss the opportunities for daily scientific interaction. However, his departure also represents an opportunity to initiate research programs in new areas which will relate to the goals of the Institute.

Dr. Stanley Barban, a member of this laboratory since 1952, has accepted an administrative position in the Office of Recombinant DNA Activities (ORDA). His past scientific achievements will stand him in good stead in his new position.

Honors and Awards

Dr. Norman Salzman continued on the editorial board of the Journal of Virology. He was appointed a member of the editorial advisory board of Biochemistry and to the scientific directorate of the "Coordinating Council for Cancer Research." He also served as Chairman of the Virus Task Force, Panel V, and was reappointed Professorial Lecturer at Georgetown University School of Medicine. He served with Dr. Roger Monier, a Fogarty Scholar, as a Co-chairman of the "Papovaviruses and their Role in Cell Transformation and Oncogenesis" meeting, held February 1-3, 1978, at the NIH.

Dr. Moss continued to serve on the editorial boards of the Journal of Virology, of Antibiotics and Chemotherapy, of Intervirology, and of Virology. He also served as a member of the Virology Task Force, and as Chairman of the Poxvirus Workshop at the 4th International Congress for Virology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00123-12 LBV

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Messenger RNA: Regulation of Synthesis, Modification, and Translation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bernard Moss	Medical Director	LBV NIAID
	Jerry Keith	Staff Fellow	LBV NIAID
	Sundararjan Venkatesan	Senior Staff Fellow	LBV NIAID
	Robert Boone	Research Associate	LBV NIAID
	Ernest Barbosa	Research Associate	LBV NIAID
	Jonathan A. Cooper	Visiting Fellow	LBV NIAID
	Claude Garon	Research Microbiologist	LBV NIAID

COOPERATING UNITS (if any)

Peter Palese, Mt. Sinai Hospital, School of Medicine

LAB/BRANCH

Laboratory of Biology of Viruses

SECTION

Macromolecular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.7	6.2	3.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our primary objective is to determine the molecular events that lead to the selective transcription of DNA and subsequent processing and translation of messenger RNA (mRNA). Poxviruses provide a unique and important system for such studies since the enzymes needed for transcription are packaged within the core of infectious virus particles. Purified viral enzymes, including a guanylyltransferase and two methyltransferases have been used to reconstruct the sequence of reactions leading to the methylated cap structure of vaccinia virus mRNA, to investigate the roles of the individual modifications on ribosome binding and protein synthesis, and for RNA sequencing. Similar and additional enzymes have been isolated from uninfected Hela cells to determine the steps involved in the processing of eukaryotic mRNA. Evidence for temporal control of vaccinia virus gene expression at the transcriptional level has been obtained by DNA:RNA hybridization studies and by translation of viral mRNA in a cell-free protein synthesizing system. A transcriptional map of the vaccinia virus genome is being prepared using DNA fragments produced by restriction endonuclease digestion. Electron

microscopic analysis of the vaccinia virus genome led to the visualization of a 10,500 nucleotide inverted terminal repetition. Preliminary studies suggest that a portion of the repeated sequence is transcribed.

MAJOR FINDINGS

1. Post-transcriptional modification of RNA.

A. Enzymes that modify the 5'-end of viral and cellular mRNAs. Viral and cellular mRNAs were shown in previous years to contain a 5'-terminal cap structure [$m^7G(5')pppN^m$] consisting of a 7-methylguanosine (m^7G) linked by a 5' to 5' triphosphate bridge to one or two consecutive 2'-O-methylated ribonucleosides (N^m). During the past year, a third vaccinia virus enzyme, an S-adenosylmethionine:mRNA (nucleoside - 2' -)-methyltransferase, involved in the formation of the cap has been extensively characterized. The enzyme specifically methylates the penultimate nucleoside of RNA ending in $m^7G(5')pppN$-. This specificity indicates that 2'-O-methylation follows guanylylation and guanine-7-methylation.

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2. Transcription of Vaccinia Virus DNA.

A. Multiple roles for ATP in the synthesis and processing of mRNA by vaccinia virus. In previous years we isolated several enzymes from vaccinia virus cores including two distinct ribonucleoside triphosphatates and a protein kinase, that have unknown biological functions. The utilization of ATP, however, is a common property of these enzymes. Adenosine (β,γ-imido) triphosphate (AMP-PNP), a compound that closely resembles ATP, but which has

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C. Inverted terminal repetition in vaccinia virus DNA. An inverted terminal repetition was observed in DNA molecules extracted from vaccinia virus. The repeated sequence was visualized by (1) nicking the hairpin loops present at the ends of vaccinia virus DNA, (ii) separating the strands of DNA by alkali denaturation, (iii) allowing the single strands to self-anneal, and (iv) examining the DNA with an electron microscope. Single-stranded circular molecules, each of which contained a duplex projection (3.54 ± 0.12 μm) representing the terminal repetition, readily formed. Similar size projections were also seen in heteroduplex structures formed by cross-hybridization of the separated strands of the 2 terminal Hind III restriction fragments. Based on contour length measurements and the electrophoretic mobility of the isolated inverted terminal repetition, a molecular weight of approximately 6.9×10^6 equivalent to about 10,500 nucleotides was estimated. Evidence was obtained from DNA:RNA hybridization studies that the terminal repetition is transcribed. Preparation of more detailed transcription maps is in progress.

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Publications:

Moss, B., Gershowitz, A., Stringer, J. R., Holland, L. E. and Wagner, E. K.: 5'-terminal and internal methylated nucleosides in herpes simplex virus 1 mRNA. J. Virol. 23, 234-239, 1977.

Wei, C. M. and Moss, B.: 5'-terminal capping of RNA by guanylyltransferase from Hela cell nuclei. Proc. Natl. Acad. Sci., U.S.A. 74, 3758-3761, 1977.

Moss, B., Keith, J. M., Gershowitz, A., Ritchey, M. B. and Palese, P.: Common sequences at the 5' ends of the segmented RNA genomes of influenza A and B viruses. Virol. 25, 312-318, 1978.

Moss, B.: Poxviruses. In Nayak, D. (ed.): The Molecular Biology of Animal Viruses, Vo. 2. New York, Marcel Dekker, 1978, pp. 849-898.

Muthukrishnan, S., Moss, B., Cooper, J. A., and Maxwell, E. S.: Influence of 5'-terminal cap structure on the initiation of translation of vaccinia virus mRNA. J. Biol. Chem. 253, 1710-1715, 1978.

Keith, J. M., Ensinger, M. J. and Moss, B.: Hela cell RNA (2'-O-methyladenosine-N^6-)-methyltransferase specific for the capped 5'-end of messenger RNA. J. Biol. Chem., in press.

Keith, J. M., Muthukrishnan, S. and Moss, B.: Effect of methylation of the N^6-position of the penultimate adenosine of capped mRNA on ribosome binding. J. Biol. Chem., in press.

Cooper, J. A. and Moss, B.: Transcription of vaccinia virus mRNA coupled to translation in vitro. Virology, in press.

Barbosa, E. and Moss, B: mRNA(nucleoside-2'-)-methyltransferase from vaccinia virus: purification and physical properties. J. Biol. Chem, in press.

Barbosa,E. and Moss, B: mRNA(nucleoside-2'-)-methyltransferase from vaccinia virus: characteristics and substrate specificity. J. Biol. Chem, in press.

Gershowitz, A., Boone, R. F. and Moss, B.: Multiple roles for ATP in the synthesis and processing of mRNA by vaccinia virus: specific inhibitory effects of adenosine (β,γ-imido)triphosphate. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI 00124-09 LBV

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Replication of the Parvovirus, KRV

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Dr. Lois A. Salzman Research Chemist LBV NIAID

Other: Dr. Tonu Wali, Visiting Fellow LBV NIAID
Ms. Phyllis Fabisch, Microbiologist LBV NIAID

COOPERATING UNITS (if any)

Dr. Darrell Campbell, Jr., National Cancer Institute

LAB/BRANCH
Laboratory of Biology of Viruses

SECTION
Biochemical Virology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been studying the specific biochemical mechanisms involved in the replication of the autonomous parvovirus, KRV in a rat nephroma cell line. The virus contains one molecule of linear single-stranded DNA and three capsid proteins. Ongoing studies involve the mechanism of viral DNA replication, the intermediates in this replication, and the proteins associated with, and possibly influencing DNA replication. We are also studying the viral-specific mRNAs and their transcription.

Project Description

During the last year we determined that the linear single-stranded viral DNA contains termini on both the 3' and 5' ends which are resistant to the single-stranded specific endonuclease S1. These termini composed of 90 to 150 nucleotides are believed to have a hairpin configuration. The hairpin at the 3' end of the molecule can serve as a self-primer for synthesis of a double-stranded linear intermediate. We have utilized the ability of the viral KRV-DNA to self-prime in the presence of the "large fragment" of E. coli DNA polymerase to synthesize a completely double-stranded molecule. We have cleaved the viral DNA at intervals during the synthesis of the duplex molecule and hydrolyzed it with restriction endonucleases. Using this procedure, we obtained restriction enzyme fragments in the order of their synthesis and have prepared a linear map of the fragments. This in vitro synthesis of pure preparations of double-stranded DNA using virion DNA and DNA polymerase and the knowledge of the sequence of restriction enzyme fragments will aid us in further studies. We hope to sequence the nucleotides at the 3' terminus and the adjacent area to gain information about the initiation sequence for DNA polymerase. From use of restriction enzyme fragments we may be able to detect and sequence the attachment site for RNA polymerase and for the mRNA.

A double-stranded DNA intermediate has been implicated in KRV-DNA replication but it is difficult to study or purify the small in vivo synthesized KRV-DNA intermediate molecules. We hope to learn more about DNA synthesis and the replicative intermediates using nuclear DNA-protein complexes capable of synthesizing DNA. Such complexes have been used to study the DNA replication of adenovirus and SV-40 virus.

We have also recently studied the synthesis of viral specific RNA in the cytoplasm, nucleus and the polysomes of KRV infected cells. We have found by DNA hybridization, two viral specific RNA species sedimenting at 22 and 12's in aqueous sucrose gradients. In the nucleus we have also seen a 26S viral RNA which would represent an entire transcript of the viral genome. We intend to investigate the possibility of post-transcriptional processing of the 26S to the functional 12 and 22S RNA's. We also wish to determine the genome location of the viral RNA's by use of in vitro restriction enzyme fragments and visualization of "R=loops" in the electron microscope. We also intend to determine the proteins coded for by the viral m-RNA's using a reticulocyte lysate technique.

In conjunction with Dr. Darrell Campbell (NCI), we have found that rat adenocarcinoma cells 13762 (a DMBA induced tumor) are strongly inhibitory to lymphocyte proliferative responses. This inhibition was due to a contamination of the line with KRV. The virus can replicate in rat lymphocytes, predominantly in lymphocytes stimulated to active proliferation. Other transplantable rat tumor lines (C58NT) D is also contaminated with KRV. The presence of such viruses in in vivo passaged tumor lines may adversely affect the measurement of lympho-proliferative responses to tumors in vitro.

Publications

1. Campbell, D. A., Jr., S. P. Staal, E. K. Manders, G. D. Bonnard, R. K. Oldham, L. A. Salzman and R. B. Herberman. Inhibition of *in vitro* lymphoproliferative responses by *in vivo* passaged rat 13762 mammary adenocarcinoma cells II. Evidence that Kilham rat virus is responsible for the inhibitory effect. Cell Immunol. 33, 378-391 (1977).

2. Salzman, L. A. and F. Koczot. Isolation of nucleoprotein from the parvovirus, KRV. Virology 84, 434-442 (1978).

3. Salzman, L. A.: The Parvovirus, p. 539-587. In D. P. Nayak (ed.) Molecular Biology of Animal Viruses, Vol. 2, Marcel Dekker, New York. 1978.

4. Salzman, L. A. and P. Fabisch. Studies on the Replication of KRV Single-stranded Linear DNA. J. Gen. Virol. 39, 571-575 (1978).

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6. Salzman, L. A., L. McKerlie, P. Fabisch and F. Koczot. Studies on a Protein Found Associated with Kilham Rat Virus. In D. Ward and P. Tattersall (eds.) Parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, L. I., New York (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00125-09 LBV

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October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mechanisms of Viral DNA Replication, Transcription, and Integration

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Norman P. Salzman	Chief	LBV, NIAID
	Walter Bruszewski	Staff Fellow	LBV, NIAID
	Minou Bina-Stein	Senior Staff Fellow	LBV, NIAID
	Nancy Chiu	Staff Fellow	LBV, NIAID
	Yaffa Reuveni	Visiting Associate	LBV, NIAID
	John T. Thompson	Staff Fellow	LBV, NIAID

COOPERATING UNITS (if any)

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Laboratory of Biology of Viruses

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Biochemical Virology Section

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NIAID, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Characterization of SV40 DNA replicating molecules is being used to determine the mechanism of replication in lytically infected African green monkey kidney cells. Studies are being carried out on the mechanism of chain elongation and to characterize the proteins associated with replicating molecules. Nascent RNA chains that are attached to template DNA molecules are being isolated and regulation of viral m-RNA transcription is being studied. Transcription complexes have been isolated from infected cells which continue viral m-RNA synthesis in vitro. Their properties give insight into the initial transcription products and they have shown that processing of viral transcripts is one mechanism that operates to regulate transcription.

SV40 DNA Synthesis

In previous studies, we have characterized intermediates in SV40 DNA replication by using DNA extraction conditions which disassociate protein from nucleoprotein complexes. In our current studies, we have extracted cells under conditions where the integrity of viral nucleoprotein complexes is maintained. These complexes can support the continued replication of SV40 DNA in vitro. Both cellular and viral chromatin can exist in at least two distinct configurations. In one form nucleosomes appear as beads on a string in a 100-120 A fiber. Alternatively, chromatin can exist as 200-300 Å fibers. We find that replicating DNA nucleoprotein complexes exist as 100-120 Å fibers with nucleosomes present on both sides of the replicating fork; and that progeny chromosomes also have this same structure. This structure is not sufficiently compact to be incorporated into virions. This implies that after completion of DNA replication, the structure must undergo additional folding prior to virion formation.

Using these replicating nucleoprotein complexes, we have re-examined why replication of progeny SV40 DNA proceeds more slowly than predicted based on the rate of chain elongation. We find that in these complexes the molecules grow to full length, but there is a block when these almost completed molecules have to undergo separation to give rise to two new daughter molecules. (Seidman, Salzman)

Transcription and Processing of Viral RNA Transcriptional Intermediates(TI's)

The levels of TI's and the specificity of the regions of the genome that are transcribed by TI's when compared with the appearance of m-RNA species in the cytoplasm provides good evidence that TI's are responsible for RNA synthesis in vivo. However, there may be a clear difference between in vivo and in vitro results when the properties of TI's are examined under conditions where only early m-RNA has been found in vivo in either the nucleus or the cytoplasm. These conditions are either at early times in a lytic cycle prior to initiation of viral DNA replication or under conditions where cells have been infected with a tsA mutant at a restrictive temperature in order to block DNA synthesis. We have examined the in vitro pattern of synthesis when cells are infected with tsA58 SV40 at a restrictive temperature and TI's are prepared 12-14 hr. post infection. Under in vitro conditions the majority of RNA species (60-70%) are made from the late strand, although RNA transcribed from the early region of the early strand is also readily seen.

Based on our data with transcriptional intermediates, we have proposed a model to explain temporal regulation of transcription. We propose that input viral DNA has two promotors and both are available. They give rise to early and late transcripts. Early transcripts code for T antigen and it suppresses the synthesis of additional T antigen. We are currently testing different predictions based on this model (Chiu, Berkenmeier and Salzman)

Studies of Viral RNA Under In Vivo Conditions

By pretreating SV40 infected BSC-1 cells with glucosamine, ^{3}H-uridine labeling of both cellular and viral RNA can be halted instantaneously by addition of cold uridine. We have studied the fate of pulse-labeled viral RNA from cells at 45 hr. postinfection under these conditions.

During a 5 min. period of labeling, both the messenger and non-messenger regions of the late strand are transcribed. After various chase periods, nuclear viral species which sediment at 19, 17.5 and 16S are observed. Of the material present at the beginning of the chase period, 50% is degraded rapidly with a half life of 8 min. (initial processing). This rapidly degraded material is that fraction of the late strand which does not give rise to stable late m-RNA species. Forty percent is transported to the cytoplasm and 10% remains in the nucleus as material which sediments in the 12-4S region. These 2-4S viral RNAs have a half life of 3 hr. and hybridization studies suggest that they are in part coded for by the late strand non-messenger region and are derived from the initial nuclear processing step.

Transport of nuclear viral RNA into the cytoplasm is detected after a 5 min. pulse and a 7 min. chase. The maximum amount of labeled viral RNA is accumulated in the cytoplasm after a 30 min. to 1 hr. chase (Chiu, Salzman).

Mechanisms of RNA Splicing

During the past two years, a series of papers have described the generation of species of m-RNA molecules which arise from two distinct and separate DNA sequences by splicing. In the case of SV40, the integrity of the coding sequence is preserved and a leader sequence is spliced to it. This mechanism of splicing is being examined by a technique which first involves the isolation of specific regions of the viral genome that are generated by restriction enzyme cleavage and are fractionated by column chromatography. These fragments are then annealed to viral m-RNA and the DNA is used as a primer for the enzyme, reverse transcriptase, and a complementary DNA copy of the m-RNA is made. This DNA is then sequenced. The sequence of the c-DNA has been compared with the known nucleotide sequence of SV40. The regions of the DNA that have been deleted, and the nucleotide sequence of the splice have been exactly determined. (Bina-Stein, Thompson, Salzman)

Structure of Early m-RNA Species

Using column chromatography and restriction enzyme cleavage of SV40 DNA radiolabeled probes of 20 to 200 nucleotides which correspond to known regions of the viral genome have been isolated. These have been hybridized to early viral m-RNA and the reaction products fractionated by velocity sedimentation. This procedure has provided direct physical evidence for two early m-RNA species that contain different spliced structures (Thompson, Salzman).

Processing of Nuclear RNA-A fraction of RNA is resistant to RNA'ase digestion. These regions presumably consist of self complementary regions and they may serve as signals in RNA processing. Since these regions are double stranded, we have used compounds which interact with double stranded nucleic acid to see how they affect RNA metabolism. We find that Proflavin blocks RNA processing, and thus its transport from the nucleus. We are currently studying how synthesis of specific RNA sequences are affected by this drug (Chiu, Bruszewski, Salzman).

Shani, M., Seidman, M. and Salzman, N. P. (1977). In vivo transcription on Oligomeric SV40 DNA. Virology 83: 110-119.

Salzman, N. P., Birkenmeier, E., Chiu, N., Radonovich, M. and Shani, M.(1977) Regulation of Transcription during the SV40 Lytic Cycle. INSERM 69: 243-252.

Monier, R. and Salzman, N. P.: Summary of the International Symposium on Papovaviruses and Their Role in Cell Transformation and Oncogenesis. Bethesda, Md., 1978.

Chiu, N., Radonovich, M., Thoren, M., and Salzman, N. P. (1978) Selective Degradation of Newly Synthesized Non-messenger SV40 Transcripts. J. Virol., in press.

Seidman, M., Garon, C. and Salzman, N. P. (1978) The Relationship of SV40 Replicating Chromosomes to Two Forms of the Non-Replicating SV40 Chromosome. Nucl. Acids Res., in press.

Panel V--Virus Task Force. (1978) N. P. Salzman, Chairman, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00126-05 LBV

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Restriction Enzyme Analysis of Vaccinia Virus DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. Frank DeFilippes Research Physicist (Gen) LBV/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biology of Viruses

SECTION

Macromolecular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of the project is to study the organization of genes in large DNA molecules obtained from animal viruses. The production and ordering of unique segments of vaccinia virus DNA provides a physical map of the genome. A transcriptional map of the genome can also be determined by use of the isolated unique DNA segments. The identification of blocks of genes which produce functionally related proteins is obtained by examining transcription, and translation of the RNA products of DNA segments larger than 10^7 daltons. The preparation of several different restriction enzymes which cut vaccinia DNA into these large segments is necessary. I am also working to improve the techniques for separating and isolating very large pieces of DNA which differ in size and base composition by relatively small amounts, and for hybridizing segments isolated after digestion with one restriction enzyme to segments produced after digestion with another enzyme.

PHS-6040

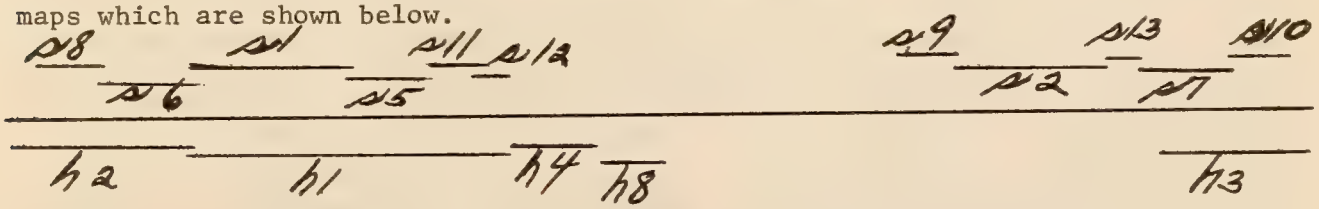
Vaccinia DNA has covalently-linked-complementary (CLC) ends which define each end of the physical map of the genome. Segments of the DNA produced by restriction nuclease digestion or mechanical shearing can be separated into CLC ends or interior segments by BND-cellulose chromatography after the segments have been alkali denatured and neutralized since this leaves only the CLC segments as duplex chains.

Restriction enzymes such as Hind III, Sal I, Xho I, Kpn I, Bgl II and Pvu II are used to digest vaccinia DNA to obtain unique DNA segments which are separated by agarose electrophoresis. To obtain a physical map segments produced by one enzyme are isolated from agarose and digested by a second enzyme to find overlapping segments. End segments, such as the 2nd and 3rd largest DNA segments produced by Hind III digestion (called h2 and h3) are identified as described. Since the Hind III ends have MWs of 1.7×10^7 and 1.3×10^7 daltons they can also be used to identify end segments resulting from digestion with other enzymes by hybridization experiments. This is necessary for Sal I digests where end fragments can not be demonstrated directly by the rapid renaturation procedure. Hybridization shows Sal segments s8 and s6 to be at the h2 end and segments s10 and s7 to be at the h3 end, where the numbers indicate segments of increasing mobility (decreasing size) in any particular electrophoretic separation. Mechanical shearing experiments place s8 outside s6 and s10 outside s7.

General shearing experiments can be coupled with the procedure to isolate CLC segments to obtain CLC fragments which are 1/4 or 1/8 etc. of the original genome length. These long fragments are then digested with a restriction nuclease to identify groups of segments which are at each end of the molecule.

A third type of experiment generates a number of reproducible partial DNA segments each of which includes several limit product segments. This results from including drugs, such as actinomycin D and distamycin, which block certain cleavage sites, in the restriction nuclease digestion reaction. Actinomycin D selectively inhibits Hind III cleavage to produce a partial segment which contains linked segments h2, h1, h4, h8. This partial has a MW of 60×10^6 daltons or 1/2 the genome length.

All these approaches have been used to generate parts of Hind III and Sal I maps which are shown below.



Different methods for extracting large DNA (10^7 daltons or more) from agarose have been examined. Ethanol precipitation was always used as a final step to concentrate DNA. The highest recovery (50-70%) of unbroken DNA suitable for redigestion with a second restriction muclease was obtained by refining a method which included dissolution of agarose containing DNA in sodium perchlorate or potassium iodide (KI) solution followed by adsorption on hydroxyapatite and desalting on Sephadex. More variable recoveries (30-65%) resulted when agarose was dissolved in KI and the DNA was recovered by banding after equi-

librium centrifugation in KI and desalted on Sephadex. The method is less efficient for recovering less than 1μg amounts and frequently the centrifugal band is contaminated by agarose.

DNA will bind to glass powder (less than 10μ) in concentrated NaI or KI solution. After sedimenting the glass the DNA is eluted by low ionic strength solution. 40-70% of the DNA was recovered in preliminary experiments but each batch of powder must be tested for its capacity since conditions to produce the powders have not been standardized. This method may not require ethanol precipitation. Also the presence of sonicated duplex DNA carrier greatly reduces the capacity of the glass for large DNA.

Electrophoretic elution of DNA in agarose followed by DEAE-cellulose chromatography gave 40-70% recovery of DNA of MW 10^7 daltons but only 30-50% recovery of DNA greater than 2×10^7 daltons.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00127-11 LBV

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Structure and Function of Genetic and Protein Components of Defective Parvoviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James Rose	Medical Officer	LBV NIAID
Other:	Mark Buller	Visiting Fellow	LBV NIAID
	Edwin Sebring	Research Chemist	LBV NIAID
	John Wang	Visiting Fellow	LBV NIAID
	Frank Koczot	Research Biologist	LBV NIAID
	Claude Garon	Research Microbiologist	LBV NIAID
	James Garrison	Bio Lab Tech (Biochem)	LBV NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biology of Viruses

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.6	3.6	3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Main objectives in studying these defective human parvoviruses (AAV) are to (i) define specific biochemical mechanisms used in synthesizing their DNA, RNA and proteins, (ii) identify and characterize the helper virus-mediated step(s) required for their replication and (iii) relate biochemical findings to normal cellular processes as well as to potentials for selective interference with both AAV and Helper virus (adenoviruses, herpesviruses) infection. Among the methods used are nucleic acid hybridizations, analytical and preparative sucrose and CsCl sedimentations, molecular cleavage with restriction nucleases, gel electrophoresis and electron microscopy.

Project Description:

(1) To study further the mechanism of viral DNA replication in vivo, AAV DNA synthesis was analyzed under conditions which restrict virus-specific protein synthesis. It was found that AAV structural protein is required for the efficient processing of AAV DNA concatemers to unit length molecules. In the presence of a protein synthesis inhibitor, cyclohexamide, or in primary monkey kidney cells in which AAV messenger RNA is not translated, high molecular weight concatemers accumulate, and single-stranded progeny molecules are not generated. These findings suggest that AAV structural protein plays more than one role in the viral replication process: (i) it may be required for specific processing of concatemers (itself an endonuclease) or, coupled with a cellular endonuclease, may simply enhance the efficiency of processing by a cellular enzyme, (ii) it may be required to prevent reinitiation of DNA synthesis on single strands displaced from replicating DNA (i.e., prevention of conversion of single strands to double-stranded replicating intermediates) and (iii) it serves to encapsidate separate plus and minus progeny strands. Because the AAV DNA replication process probably shares much in common with that of the cell, definition of specific requirements for AAV DNA synthesis should provide insight into the mechanism of cellular DNA synthesis. We plan to continue to study these requirements with this latter objective in mind.

(2) The enzyme(s) responsible for AAV concatemeric processing may also play a role in normal cellular DNA synthesis. We have now purified two single-strand-specific endonucleases from KB cells as possible candidates for such processing enzymes. The first enzyme has a pH optimum at 9.2, requires Mg^{2+} for activity, and is inhibited by mono-or divalent cations. Its sedimentation coefficient of 4.6S is based on sucrose gradient sedimentation, and it has a molecular weight of 54,000 as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme specfically catalyzes the endonucleolytic cleavage of denatured DNA, yielding acid-soluble oligonucleotides which contain 5' - phosphoryl termini. The rate of hydrolysis of poly(dT) is approximately eight-fold greater than that observed with denatured DNA, although the K_m for both substrates is 1.74×10^{-5}M. The relative rates of hydrolysis of homopolymers by the endonuclease are poly(dG)>poly(dT)>poly (dA)>poly>(dC). Purified enzyme preparations also hydrolyze poly(U), releasing acid-soluble products. This activity cosediments in sucrose gradients with the DNA endonuclease activity, suggesting that both activities are contained in the same enzyme molecule. The second enzyme has a pH optimum at 7.4 and hydrolyzes poly(dT) relatively faster than the other homopolymers. This enzyme appears to have a high degree of sequence specificity. Efforts will be continued to attempt to identify and characterize enzymes involved in virus and cellular DNA synthesis.

(3) To investigate the basis of the AAV multiplication block in herpesvirus (HSV)/AAV-infected cells, quantitative and kinetic parameters of AAV DNA, RNA and protein synthesis were compared in HSV/AAV and adenovirus(Ad)/AAV-infected KB cells. Several distinct differences were found. In HSV/AAV infections the intervals between virus addition and detectable initiations of AAV-specific DNA, RNA and protein synthesis were substantially diminished, and the maximal rate of synthesis in each case was maintained for a shorter period of time. Also, AAV RNA synthesis commenced simultaneously with AAV DNA synthesis

in contrast to the delayed onset of AAV RNA synthesis in Ad/AAV infection. This RNA, however, exhibited a shorter half-life in the cytoplasm of HSV/AAV-infected cells. Finally, electron microscopic and hybridization studies detected significant quantities of progeny AAV particles in isopycnic gradient analyses of HSV/AAV-infected cultures although approximately 1000-fold lower than in comparable Ad/AAV infections. These data indicate, for the first time, that HSV is capable of acting as a complete helper for AAV multiplication, but that this capability is relatively inefficient. Thus, herpesviruses appear to possess the ability to perpetuate naturally defective parvoviruses. This may explain, in part, the latent persistence of AAV in certain cells, and is a fact that should be considered in the preparation of some vaccines.

(4) To test whether Ad functions expressed in malignantly transformed cells were capable of inducing AAV replication, AAV infection of Ad-transformed human kidney cells was investigated. The transformed cell line synthesized T antigen(s) as well as 72K DNA-binding protein. Synthesis of AAV nucleic acid could not be detected in the absence of added helper virus (Ad 5). Therefore, in this cell line (as well as in a rodent line we previously tested) the Ad requirement(s) for AAV replication was not synthesized. Thus, Ad DNA-binding protein and those proteins mapping within the Ad transforming sequences (first 5-10% of the left end of the genome) do not assist AAV replication (at least without some other adenovirus protein not expressed in the transformed cells). We plan to continue studies directed at identifying and isolating the specific AAV helper factor(s) supplied by both adenoviruses and herpesviruses. Results from our previous studies indicate that this factor(s) may be involved in initiation of AAV DNA synthesis. Confirming and understanding its role in initiation of AAV DNA synthesis should provide insight into the mechanism of initiation of adenovirus and herpesvirus DNA synthesis. This kind of information is relevant to possible selective interference with virus infection.

Publications:

Straus, S. E., Sebring, E. D. and Rose, J. A.: Self-primed replication of adenovirus-associated virus DNA. In Ward, D. and Tattersall, P. (Ed.): Cold Spring Harbor Symposium on Parvoviruses. New York, Cold Spring Harbor, 1978, pp. 275-280.

Buller, R. M. L. and Rose, J. A.: Characterization of AAV polypeptides synthesized in vivo and in vitro. In Ward, D. and Tattersall, P. (Ed.): Cold Spring Harbor Symposium on Parvoviruses. New York, Cold Spring Harbor, 1978, pp. 281-288.

Bloom, M. E. and Rose, J. A.: Transcription of adenovirus-associated virus RNA in isolated KB cell nuclei. Virology 84: 118-126, 1978.

Buller, R. M. L., and Rose, J. A.: Characterization of AAV-induced polypeptides in KB cells, J. Virol. 25: 331-338, 1978.

Wang, E. C., and Rose, J. A.: Purfication and characterization of a DNA single strand-specific endonuclease from human cells. Biochemistry 17: 544-549, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00128-11 LBV

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Properties of Adenovirus DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigators:	Claude F. Garon	Research Microbiologist	LBV	NIAID
	James A. Rose	Medical Officer	LBV	NIAID
Other:	Ronald P. Parr	Biologist	LBV	NIAID
	James W. Garrison	Bio Lab Tech(Biochem)	LBV	NIAID

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LAB/BRANCH

Laboratory of Biology of Viruses

SECTION

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NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	.4	.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of these studies has been to obtain a basic understanding of the structural arrangement of specific viral DNA sequences within several groups of oncogenic and nonocogenic human adenoviruses. A number of unique features have been described which may have implications in viral replication or carcinogenesis or both. However, also central to these studies has been the development of a technology which would allow the collection of preparative amounts of both physically and genetically defined fragments of DNA with the ultimate goals of (1) relating these DNA sequences to those biochemical activities which permit these viruses to influence or gain control of cellular functions (e.g., cell lysis and transformation and (2) of defining basic mechanisms for regulation o genetic expression (e.g., initiation of DNA synthesis).

The structure of Ad 18 DNA from two plaque isolates derived from the prototype strain has been analyzed by cleavage with several restriction endonucleases and by electron microscopic heteroduplexing techniques. Fragment sizes were determined by contour length measurements, sucrose gradient sedimentation and agarose gel electrophoresis. A physical ordering of fragments was obtained by comparative digestion of isolated fragments and heteroduplex mapping. End fragments were identified by their susceptibility to exonuclease digestion and by the presence of covalently attached terminal proteins that remain when viral DNA is released from virions in sucrose-guanidinium gradients. It was found that plaque 2 DNA molecules were about 4% longer than those of plaque 1. In addition, the inverted terminal repetition of plaque 2 DNA was 3-4% of genome length based on measurements of duplex "panhandles" generated by self-annealing of single DNA strands. A visible "panhandle" could not be detected after self-annealing of single-stranded plaque 1 DNA. Furthermore, restriction enzyme cleavage patterns of the two plaque isolates appeared to differ only in the size of the terminal fragments. Although these findings indicate that the greater length of plaque 2 DNA results from a longer inverted repetition, plaque 1:2 heteroduplex molecules suggest that an additional structural difference might also exist at the opposite molecular end. In contrast to previous reports of Ad 18 isolates in which enlargement of terminal sequences appear to occur in place of internal DNA sequences, these data provide evidence for a new arrangement in which sequences are added to the termini of DNA molecules with no other apparent internal molecular deletion or rearrangement. Of special interest is whether this Ad 18 genome contains a repetition of oncogenic sequences within the terminal insertion. If so, a "double dose" of transforming genes would be present and could account for the high oncogenic potency of this virus.

Robinson et al. (Virology 56, 54) originally reported that when adenovirus DNA was extracted from virions with guanidinium chloride, circular molecules could be detected by electron microscopy. It was proposed that these structures were formed as a result of a protein bound tightly to the two ends of the DNA molecule. Subsequent studies demonstrated the presence of a covalently attached, 55,000 M.W. protein. These terminal protein-protein interactions could be inhibited by treatment with SDS or a protease, resulting in linear, monomeric DNA. Brown et al. (Journal of Virology 16, 366) first demonstrated that these DNA-protein complexes did not migrate into agarose gels during electrophoresis. This observation provided the basis for a useful means for detecting not only the presence or absence of terminal protein components but the identification and purification of terminal DNA sequences as well. We have fractionated the Ad 2 DNA-protein complex on hydroyapatite columns and analyzed the DNA eluate by agarose gel electrophoresis. Although the initial DNA-protein complexed did not migrate into agarose gels, > 75% of DNA molecules in the DNA eluate were now able to enter the gels in spite of the absence of protease or SDS treatment. When the protein in the DNA eluate was labeled in vitro with ^{125}I, protein was not seen associated with the DNA that migrated into the gels. When the DNA eluate was examined in the electron microscope, DNA was found to be essentially linear. The addition of a DNA-free protein fraction to either the DNA eluate or to phenol extracted DNA produced circularization of about 20% of these linear molecules. We conclude that circularization of the adenovirus DNA-protein complex as well as

its inability to migrate into agarose gels is likely due to an aggregate of a virion component(s) in addition to the covalently-attached 55K protein previously described.

Recently a category of chemical compounds (psoralen and its derivatives) has generated considerable interest by its ability to penetrate intact cells and viruses without detectable structural alterations, to intercalate into the double helix of nucleic acid molecules and upon photochemical activation form covalent cross-links. The reaction is highly specific. The interstrand bonds are exceptionally stable. And, unlike other cross-linking procedures, it introduces little or no damage to the DNA molecules involved. We have attempted to apply this reagent to purified adenovirus particles in search of proteins within the virion which, by virtue of their intimate association with the DNA, might interrupt the cross-linking function of the drug and thus be detectable in the electron microscope as site-specific denaturable regions. Preliminary data would indicate: (1) the effectiveness of the drug at cross-linking deproteinized, adenovirus DNA *in vitro*; (2) the possible identification of at least two major protein interaction sites along the adenovirus serotype 2 DNA molecule; and (3) the usefulness of the drug as a probe for freezing the secondary structure of the DNA molecules both *in vivo* and *in vitro* under various conditions of temperature and ionic strength. These sequences may represent important control elements in the expression or packaging of the adenovirus genome.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00156-03 LBV

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Structural Characterization of DNA Virus Genomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Claude F. Garon, Research Microbiologist LBV NIAID

Other: Ronald P. Parr, Biologist LBV NIAID

COOPERATING UNITS (if any)
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Dr. H. B. Bradford, Louisiana Health and Human Resources Administration New Orleans, Louisiana

LAB/BRANCH
Laboratory of Biology of Viruses

SECTION
Macromolecular Biology Section

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NIAID, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structural characteristics determined in our initial studies tend to place the Molluscum contagiosum virus (MCV) genome among other members of the poxvirus group in terms of genome molecular weight and organization. The apparent denaturability of the MCV genome into a continuous, single-stranded circle would point to the presence of terminal cross-links in the DNA molecule. So far, this structural arrangement appears to be unique to members of the poxvirus group. Furthermore, genome comparison studies of eleven independently isolated virus samples using techniques of electron microscopy and agarose gel electrophoresis revealed only three genetic types of MCV. The objective of these studies has been to define these structural features of the DNA molecules and to relate them to the biochemical events involved in the replication and growth of this virus in the cells they infect.

FWS-6040

Project Description

Molluscum contagiosum virus (MCV) is a member of the mammalian poxvirus subgroup which causes a benign tumor of the skin in humans. The disease is most common in children. Cases appear to occur world-wide and are often associated with poverty, overcrowding and poor hygiene. Molluscum contagiosum virions were isolated from clinically typical skin lesions and the DNA extracted and characterized using techniques of electron microscopy and agarose gel electrophoresis. In some experiments, a comparison of the structural features of the MCV genome and other members of the poxvirus group was made.

The genome of Molluscum contagiosum virus appears in an electron microscope as a linear, duplex DNA molecule having a mean contour length of 53.02 1.87μm. Vaccinia virus DNA, similarly processed and used for comparison, measures 53.03 $\pm$ 2.2 μm. The average molecular weight of MCV-DNA was calculated to be 118 x 10^6 and appeared to be extremely sensitive to both mechanical shear and nuclease damage. Single-stranded circles measuring twice the length of linear molecules were observed with both poxvirus DNA's following high levels of denaturation. However, partial denaturation profiles of MCV and vaccinia virus DNA differed markedly when mounted for microscopy under similar conditions. MCV-DNA consistently required an additional 10° equivalents to achieve comparable levels of denaturation. Of special interest was the fact that the most easily denaturable regions of the MCV genome appeared to be the ends of the molecule.

When Molluscum contagiosum virus DNA was cleared by restriction endonuclease BAM HI, 12 fragments were produced ranging in size from 4 x 10^5 to 31 x 10^6 daltons as determined by agarose gel electrophoresis and contour length measurements. Vaccinia virus DNA, similarly treated, produced more than 23 fragments which did not appear to correspond in size to those of MCV. With the aim of comparing the genetic and structural heterogeneity of the MCV genome, eleven independently isolated virus samples were detected, with some fragments present in all three profiles, comigrating in agarose gels. Although we have not yet collected a large enough body of data to make any reasonable assessment of the relationship between the clinical characteristics of the disease and the restriction endonuclease cleavage patterns, it may be possible once the data are accumulated, to relate these observations and to develop a useful molecular epidemiological scheme. Such a scheme would make possible rapid identification and classification of MCV isolates without the necessity of propagating the virus in the laboratory. Furthermore, since the degree of sensitivity of such a system is limited only by the number of cleavage sites recognized by a given restriction enzyme, other available enzymes may easily be substituted.

We propose to continue to define structural features of these poxvirus DNA molecules with the aim of (1) comparing genetic variation among members of this group, and of (2) relating these features to those biochemical events which are involved in the replication of these agents.

Publications

Parr, R. P., Burnett, J. W. and Garon, C. F. (1977) Structural Characterization of the Molluscum contagiosum Virus Genome. Virology 81: 247-256.

LABORATORY OF MICROBIAL IMMUNITY
1978 Annual Report
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PHS-NIH
SUMMARY STATEMENT

ANNUAL REPORT OF THE LABORATORY OF MICROBIAL IMMUNITY
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
October 1, 1977 to September 30, 1978

Richard Asofsky, M.D.
Chief, Laboratory of Microbial Immunity

Research in this Laboratory concerns the basic mechanisms controlling cellular and humoral immunity to microbial and tissue antigens. A wide range of microbes, including several viruses, blood parasites, and pneumococcal polysaccharides are used in this work. Tissue antigens include constituents of integrated leukemia viruses which are present on cell surfaces, and histocompatibility antigens. The latter are studied in graft-versus-host and allograft reactions. Basic research is done on control of immunoglobulin synthesis and synthesis and assembly of immunoglobulins in plasma cells and B lymphocytes, using cloned tumor cell lines. Heterogeneity of thymus-derived cells is analyzed by use of alloantigenic markers. In the last several years, much of the work in the Laboratory has acquired a genetic base, with studies of genes controlling expression of leukemia antigens, those controlling T-lymphocyte differentiation and those controlling antibody formation to pneumococcal polysaccharide. Somatic cell hybridization is being used to probe the repertoire of some antibody responses and to understand B cell differentiation.

I. SELECTIVE IMMUNOSUPPRESSIVE EFFECTS OF HYDROCORTISONE.

Hydrocortisone and other related steroids have long been used as immunosuppressive agents in the treatment of several immunological disorders. Massive lysis of lymphocytes often occurs after steroid treatment; but, such lysis is not demonstrable in all animal species and different mechanisms have been evoked to account for the immunosuppression noted in "steroid-resistant" species. Although thymus-derived (T) lymphocytes are generally considered to be more steroid-sensitive than bone marrow-derived precursors of antibody-forming cells (B cells), T cells are now classified in terms of functional subpopulations capable of suppressing, amplifying, or helping immune response. This makes it difficult to determine which cell population is critically sensitive to steroid. We have examined the *in vivo* sensitivity to hydrocortisone succinate of suppressor, amplifier, and helper T cells with reference to the antibody response of mice, a steroid sensitive species, to Type III pneumococcal polysaccharide (SSS-III). Both the generation and expression of helper T cell activity was either completely eliminated or substantially reduced after steroid treatment; helper T cell activity is therefore steroid sensitive. By contrast, neither amplifier nor suppressor T cell activity was influenced by steroid treatment and treatment with steroid did not alter the capacity of B cells to give an antibody response to SSS-III. These finding suggest that the immunosuppressive effects of steroid may be due both to the removal of helper T cell activity needed for the initiation of immune responses to some antigens, and the sparing of

suppressor T cells which normally act to limit the magnitude of antibody responses produced after immunization. (Dr. P. J. Baker; Dr. R. B. Markham; Dr. B. Prescott; Mr. P. W. Stashak, and Ms. D. F. Amsbaugh, LMI/NIAID).

II. EXPRESSION AND GENETIC CONTROL OF XENOTROPIC gp70 ANTIGEN (XenCSA) ON LYMPHOCYTES STUDIED.

Antisera from rabbits immunized with rabbit corneal (SIRC) cells infected with xenotropic murine leukemia viruses (MuLV) were tested for their reactivity with MuLV and the surface of cells exogenously or endogenously infected with MuLV. The sera demonstrated class specificity for xenotropic MuLV in that they neutralized xenotropic viruses isolated from several strains of mice but did not neutralize ecotropic or amphotropic MuLV. SDS-PAGE analyses of precipitates made from disrupted radiolabeled MuLV showed predominant reactivity with gp70 determinants although the sera also recognized p30, p15 and p12. Only molecules with characteristics of gp70 were precipitated out of radiolabeled antigens from the surface of cell lines exogenously infected with MuLV or from lymphocytes of normal mice. Cross reactivity between virion envelope and cell surface antigens was demonstrated by absorption testing. The set of cross-reactive antigens present on the envelopes of xenotropic MuLV and murine lymphocytes was termed XenCSA for xenotropic MuLV envelope-related cell surface antigen.

XenCSA expression on thymocytes and spleen cells from 63 strains of inbred mice was assessed by immunofluorescence using flow microfluorometry. Lymphocytes from both tissues of all strains expressed XenCSA but to a highly variable extent. Based on mean fluorescence values, thymocytes and spleen cells from each strain were classified as being high or low for XenCSA. There was a high association for expression of XenCSA and G_{IX} on thymocytes but no correlation with Fv-1, T1a, H-2, Pca-1 or GCSA. The level of XenCSA expressed on the cell surface was also independent of the amount of infectious xenotropic MuLV produced by those cells. (Drs. H. C. Morse III; B. J. Mathieson; J. W. Hartley (LVD); T. M. Chused (NIDR); M. Boehm-Truitt (NIDR), and Ms. S. O. Sharrow (NCI).

Flow microfluorometry was used to assess levels of XenCSA expressed on lymphocytes of mice derived from crosses between C57BL/6 (B6) and DBA/2 (D2) including 24 recombinant inbred strains (BXD RI's). The results suggest that XenCSA expression is affected by more than one gene but that the predominant influence is exerted by a single semidominant gene apparently located on chromosome 4 at or in close proximity to the Fv-1 locus. Studies of spontaneous virus production in $B6D2F_1$ X D2 mice suggest that this locus may also affect production by spleen cells of xenotropic MuLV registering in a fluorescent antibody assay of mink lung cells. It is possible that the localization of this locus to chromosome 4 represents quasi rather than true linkage. (Drs. H. C. Morse III; B. J. Mathieson; J. W. Hartley (LVD); T. M. Chused (NIDR); Ms. S. O. Sharrow (NCI), and Dr. B. Taylor (The Jackson Laboratory).

III. SPONTANEOUS B CELL LYMPHOMAS ESTABLISHED IN TISSUE CULTURE SYNTHESIZE 8S IgM.

Spontaneous lymphomas of BALB/c mice, both in vivo tumors and cell lines established in long term tissue cultures, were investigated for their ability to synthesize IgM using radiolabelled amino acid precursors. Immunoglobulins manufactured by lymphomas K46 and L10A had the molecular weights of monomeric IgM and IgM half molecule. Both of these molecules could be immunoprecipitated with class specific anti-IgM but not anti-IgA or anti-IgG. When precipitated with polyvalent anti-Ig L10A synthesized monomeric immunoglobulins which migrated as two peaks in contrast to their single counterpart precipitated with anti-IgM. The second peak migrated in the region expected for IgD. Monomer and half molecules were composed of similar ratios of μ-chains to light chains linked by disulfide bonds. The μ_2L_2 monomer of these B cell lines migrated slightly slower in SDS PAGE than a μ_2L_2 secreted by a myeloma. Thus, these lymphomas synthesize immunoglobulins with the chemical and antigenic characteristics typical of monomeric membrane-attached IgM and IgM half molecules, plus a molecule resembling IgD on L10A only. Lymphoma assembly of monomeric IgM may follow the same initial biosynthesis sequence as myeloma assembly. (Drs. P. E. McKeever, K. Jin Kim; R. Asofsky, and R. Merwin (NCI), Mr. G. B. Nero (Student).

IV. RELATIONSHIP BETWEEN Fc RECEPTORS AND Ia, OR Mls-ASSOCIATED ANTIGENS ON THE SURFACE OF B LYMPHOMA LINES.

A panel of spontaneous BALB/c B cell lymphoma lines with different cell surface phenotypes were used to study the relationship of Fc receptors, Ia and Mls-associated (tentatively called LyM) antigens. These cultured lymphomas have the following B cell characteristics: They a) express surface Ig; b) have Fc receptors tested by sheep erythrocyte-antibody rosetting assay; c) do not ingest latex particles, and d) are negative for esterase activity.

One of these cell lines, A-20, is Ia^+, Fc^+, LyM^+; another M 12, is Ia^-, Fc^+, LyM^+. Using these and other lines, we have shown that Fc receptors on Ia^+ cells (e.g. A-20) can be inhibited by anti-Ia sera. There is not such inhibition on Ia^- cells (e.g. M12). However, anti-Mls serum can inhibit Fc rosetting on tumors with either Ia^+ or Ia^- phenotype.

Thus, we can demonstrate inhibition of Fc receptors with anti-Ia sera similar to that reported for normal spleen cells. These data also suggest that cell surface interactions between Mls-associated antigens and Fc receptors can occur in the absence of antigens coded for in the I region of the H-2 complex. (Drs. C. Kanellopoulos-Langevin, K. Jin Kim, D. H. Sachs (NCI), and R. Asofsky).

V. DIFFERENTIATION OF B-CELLS STUDIED BY SOMATIC CELL FUSION.

Murine B-lymphoma cultured cell lines bearing membrane IgM and lacking the ability to secrete measurable amounts of IgM, were fused with drug resistant cell lines derived from the IgG_{2b} producing MPC-11 myeloma. Many of the hybrid clones synthesized and secreted large amounts of IgM, as judged by radial and double immunodiffusion in agar of culture supernatants

and by polyacrylamide gel electrophoresis of biosynthetically labelled IgM. When fused with an IgG_{2b} producing myeloma, many of the hybrids also produced IgG_{2b} indicating that there was no suppression of the parental IgG synthesis in the hybrids. The amount of IgM and/or IgG_{2b} secreted by the hybrids was higher than that secreted by myeloma cell lines. The synthesis of the γ_{2b} heavy chain of the MPC-11 myeloma, was not reexpressed by fusion of a γ_{2b} non-producer myeloma cell variant with B-lymphoma. No other classes of Ig heavy chains, beside the γ_{2b} and the μ chains, was found to be secreted by any of more than 100 B-lymphoma x myeloma hybrids examined. The results of the present work suggest that myeloma cells can fuse not only with normal plasma cells but also with less mature normal B-lymphocytes and that this fusion "induces" the maturation of B-lymphocytes into IgM secreting cells. (Drs. R. Laskov; K. Jin Kim, and R. Asofsky, LMI/NIAID).

Administrative:

Very successful years were spent in the Laboratory by Dr. Reuven Laskov a Visiting Scientist and Dr. Dan Eilat a Visiting Associate from Jerusalem. They engaged mainly in research on somatic cell hybridization. Dr. Isaac Betel from the Radiobiological Institute, TNO, The Netherlands, joined Dr. Bonnie Mathieson as a guest worker from January through July. They analyzed differentiation among thymocytes, using flow microfluorometry on cells separated by density and by mass. The Laboratory was joined by Dr. Philip Munoz, a Research Associate. A fluorescence-activated cell sorter and a PDP 11/34 was bought to be shared by intramural scientists. Dr. Thomas Chused, NIDR, will join LMI in the fall to help superintend the operation of the sorter, and to continue his work on autoimmune diseases.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00134-16 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Control of Immunoglobulin Synthesis in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Asofsky	Laboratory Chief	LMI/NIAID
Other:	R. Laskov	Visiting Scientist	LMI/NIAID
	D. Eilat	Visiting Associate	LMI/NIAID
	P. E. McKeever	Research Associate	LMI/NIAID
	K. J. Kim	Visiting Fellow	LMI/NIAID
	C. Kanellopoulos-Langevin	Visiting Fellow	LMI/NIAID
	Y. J. Rosenberg	Visiting Fellow	LMI/NIAID
	B. J. Mathieson	Staff Fellow	LMI/NIAID
	W. J. Logan	Biological Laboratory Tech	LMI/NIAID
	C. B. Evans	Biological Laboratory Tech	LMI/NIAID
	B. J. Fowlkes	Microbiologist	LMI/NIAID
	R. Merwin	Biologist	VO/NCI
	D. H. Sachs	Senior Investigator	IB/NCI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Experimental Pathology Section

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NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Neoplastic lymphoid cells of B-cell and T-cell type were maintained in long term transplant. The T cell lines showed a variety of Lyt 1, Lyt 2, and TL phenotypes, and an 8-fold range of terminal deoxynucleotidyl transferase. The B cell lines were Ig^+, Fc^+, usually Ia^+, and LyM^+. Anti Ia or anti LyM could inhibit Fc rosettes.

Somatic hybridization of B lymphomas with drug-marked MPC-11. Hybrids showed greatly amplified synthesis and secretion of pentameric IgM.

Somatic hybrids between MPC-11 and spleen cells from NZB x NZW F_1 mice yielded hybrid clones, one of which secreted IgG_3 antibody to ribosomal RNA, which did not bind to DNA.

Project Description:

Progress this year was almost entirely confined to the use of lymphoid tumors as models of normal lymphoid function. This was divided into (a) establishment and analysis of T-lymphocyte cell lines; (b) establishment and analysis of B-lymphocyte cell lines; (c) analysis of immunoglobulin assembly in SAMM 368, a plasmacytoma secreting both IgA-κ and IgG_{2b}-κ, but with chemically different κ chains, and (d) somatic cell hybridization of B cell tumor lines with an established plasmacytoma line.

Work was also done on the ontogeny of antibody and nonspecific immunoglobulin production following immunization with various T-cell dependent antigens.

(a) T-cell lymphomas, established last year, and of restricted Lyt phenotypes were analyzed further. There are 10 lines, all derived from BALB/c AnNΠ mice, and all $Thy\ 1.2^+$ by cytotoxicity and absorption. Two lines are $Lyt\ 1.2^+$, $Lyt\ 2.2^\mp$, and $TL.2^\mp$; one is $Lyt\ 1.2^\mp$ $Lyt\ 2.2^-$, $TL.2^-$; seven are $Lyt\ 1.2^-$, $Lyt\ 2.2^+$, $Tl.2^\mp$. All of the lines contain terminal deoxynucleotidyl transferase (TdT) in levels seen in normal thymus to 8 times that level. Levels of TdT are stable in serial transfer and independent of cell surface Lyt phenotype, proving that all of these lines derive from immature thymocytes. (Drs. Kim, Mathieson, and Asofsky)

(b) Last year's report described 50 B-cell (surface immunoglobulin$^+$ (Ig^+) which were also $Fc^\mp$ and $Ia^\mp$. Six in vitro lines have been established in long term culture. All but one, M-12, are Ia^+ for all subregions by indirect fluorescence, cytoxicity, and absorption analysis. All bear Fc receptors, none C3 receptors. None ingest latex particles and none contain esterase activity. Four lines are polyvalent Ig^+, $IgM^\mp$, 2 are polyvalent Ig^+ IgM^-. Fc receptors were detected using sheep red blood cells coated with mouse IgG antibody in agglutinating amounts. A new antiserum, which reacts with products of the Mls locus of BALB/c mice (tentatively called anti LyM^- gave positive reactions with all 6 lines. Fc rosetting was inhibited with anti-Ia (ATH anti ATL) but antisera to subregions did not inhibit, probably because of low titer. Anti Mls inhibited Fc rosettes in all lines including M-12, in which anti Ia did not inhibit. Anti H-2d gave partial inhibition. These data show that inhibition of Fc binding with anti Ia sera similar to that seen with normal spleen B cells occurs. They also suggest that anti "LyM" can occur in the absence of Ia antigens. (Drs. Kanellopoulos-Langevin, Kim, Sachs (NCI) and Asofsky).

(c) Work on SAMM 368 continued from last year. Synthesis of J chains by the tumor cells was demonstrated. There is still difficulty in identifying the two separate κ chains synthesized by the cells. (Dr. McKeever)

(d) Each of the B cell lines (6) was hybridized to MPC 11 clones which have been made drug sensitive, and hybrid clones were isolated in medium containing drugs that would not support MPC-11 nor the B cell alone. In each case clones were obtained in which there was amplified synthesis of

IgM which was secreted as a pentamer. Hybrid clones transplanted to BALB/c mice produced tumors which secreted IgM in greater amounts than several myelomas which produce IgM; and more IgG_{2b} than the parent MPC-11. No other Ig heavy chains were detected. (Drs. Laskov, Kim and Asofsky)

The B cell lines mentioned above were labeled with I^{125}, using a cell surface labeling method. Surface Ig consisted of IgM and IgD-like molecules. A similar pattern was seen in Ig labeled biosynthetically with H^3-leucine. (Drs. Laskov and McKeever)

MPC-11 was hybridized to spleen cells from NZB x NZW F_1 hybrid mice which were making antibodies to nucleic acids. One of about 100 hybrids analyzed was making antibody to ribosomal RNA but not to DNA in either single or double stranded form. This antibody belonged to the unusual IgG_3 class. The hybrid has been successfully transplanted in mice. (Drs. Eilat and Laskov)

(e) The specific and nonspecific components of the immune response to sheep red blood cells and human IgG have been studied as a function of age. Nonspecific Ig formation exceed specific by more than 10-fold, as judged by class-specific "reverse plaque" formation. Differences were seen from one week of age to maturity. (Dr. Rosenberg)

Publications:

Kim, K. J., Weinbaum, F. I., Mathieson, B. J., McKeever, P. E., and Asofsky, R.: Characteristics of BALB/c T cell lymphomas grown as continuous *in vitro* lines. *J. Immunol.* 121: 339-344, 1978.

Schwartz, B. D., Campbell, P. S., Asofsky, R., Greenberg, R. S., and Zatz, M. M.: Synthesis and characterization of membrane immunoglobulin, Ia, and H-2 molecules of $Thy\text{-}1^+$ AKR/J lymphomas. *J. Immunol.* 119: 1706-1710, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AI 00135-04 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Properties of Immunoglobulin Secreting Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. C. Morse III	Senior Investigator	LMI/NIAID
Other:	Richard Asofsky	Laboratory Chief	LMI/NIAID
	M. Neiders*		
	J. Goode	Biologist	LMI/NIAID
	M. Potter	Head, Immunochemistry Sect.	LCBGY/NCI
	S. Rudikoff	Senior Investigator	LCBGY/NCI
	A. Lawton III**		
	M. Weigert***		

COOPERATING UNITS (if any)

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** Department of Pediatrics, University of Alabama in Birmingham, Alabama
*** Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania

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Laboratory of Microbial Immunity

SECTION

Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Samm 368 is a plasmacytoma of BALB/c mice previously shown to produce two classes of immunoglobulin which do not share idiotype. The tumor has been adapted to in vitro conditions and cloned. The majority of clones continue to produce both immunoglobulin classes (IgG2b and IgA) but variant clones producing only one class were detected. Cyanogen bromide fragments of the IgG2b heavy chain have been partially sequenced. The sequences define this protein as being an IgG2b inspite of the fact that it does not carry appropriate allotype determinants for this immunoglobulin class.

NZB plasmacytomas with products reactive with antisera specific for IgA and IgG3 were studied. Both determinants of these reactions appear to reside on a single molecule.

PHS-6040

Project Description:

These studies are designed to investigate the mechanisms regulating immunoglobulin (Ig) gene expression in Ig producing cells. They are based primarily on evaluations of murine plasmacytomas induced in the following mouse strains.

(1) BALB/c. Two plasmacytomas, SAMM 368 and TEPC 609, induced in this strain have been shown to produce 2 immunoglobulin (Ig) classes from single cells. Both tumors have been adapted to in vitro growth and cloned. The majority of SAMM 368 clones produced both Ig classes but variant clones producing only IgG2b or IgA were described. Chromosome analyses of SAMM 368 in vitro demonstrated that the modal number was like that of "single" producers, suggesting that the tumor is not a fusion product.

Attempts to analyze the amino acid sequences of the heavy chains from these tumors have been complicated by the finding that all four heavy chains are blocked at the amino terminus. Cyanogen bromide fragments of the IgG2b heavy chain of SAMM 368 have been isolated and partially sequenced. Although this heavy chain does not carry allotypic determinants characteristic of BALB/c IgG2b molecules, the amino acid sequence of the C-terminal fragment from this heavy chain clearly defines it as an IgG2b protein. Work is in progress to compare heavy chain fragments from MPC11, an IgG2b with appropriate allotype markers, and SAMM 368, to determine which portion of the heavy chain bears the allotypic determinants and perhaps gain further insight into their chemical nature by comparisons of sequences.

(2) NZB. More than 1200 NZB plasmacytomas have been analyzed for various characteristics. In comparison to BALB/c tumors, the frequency of IgA producers is much reduced and that of IgG's significantly increased. The frequency of products binding carbohydrate antigens was reduced 10-fold in comparison to the BALB/c series.

Three tumors were detected which carry both IgA and IgG3 Ig determinants on their products. In all 3 instances, both determinants appear to reside on single 7S molecules. Current work is directed at determining if this antigenic complex defines a new Ig isotype or reflects a recombination event within the heavy chain locus.

Almost 10% of NZB tumors produced more than one paraprotein in the primary ascites. 25 tumors were analyzed by serial transplantation and none made more than one Ig product.

(3) (BALB/c x NZB)F_1. A large number of tumors were induced in F_1 mice in hopes of obtaining a "double producer" to examine the question of allelic exclusion or inclusion in cells making more than one Ig product. No tumors producing more than one Ig class on serial transplantation were detected.

Publications:

Neiders, M. E., Morse, H. C. III, Lawton, A. R. III, and Asofsky, R.: Murine plasma cells secreting more than one class of immunoglobulin heavy chain. III. Immunoglobulin production by established cultures and cloned lines of SAMM 368. J. Immunol. 119: 457-464, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00136-06 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Functional Activities of Subpopulations of Thymus-Derived Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. J. Mathieson	Staff Fellow	LMI/NIAID
Other:	R. Asofsky	Laboratory Chief	LMI/NIAID
	S. O. Sharrow	Chemist	IB/NCI
	I. Betel	Guest Worker	LMI/NIAID
	K. Bottomly	NIH Fellow	LI/NIAID
	P. Campbell (Deceased)	Chemist	LMI/NIAID
	M. Potter	Chief, Immunochemistry Sec.	LCBGY/NCI
	R. Riblet*		
	F-W. Shen**		
	E. A. Boyse**		

COOPERATING UNITS (if any)
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A subpopulation of normal thymocytes has been demonstrated which expresses a differentiated Lyt antigen cell surface phenotype similar to that of functional peripheral T cells. Cells with the phenotype Lyt 1^{+},23^{-} represent about 10% of normal thymocytes and are increased in vivo cortisone treatment to approximately 50%.

Thymocytes can be separated by sedimentation velocity, density in BSA gradients, and peanut lectin agglutination. These separations can enrich for thymocytes of different Lyt phenotypes. A light density, large lymphocyte subpopulation can be obtained which expresses more Lyt 1 per cell than the majority of thymocytes and which is Lyt 23^{-}. The majority of these cells do not appear to be agglutinated by peanut lectin and correspond to the cells obtained after in vivo cortisone treatment.

Project Description:

I. (A) Expression of T-cell Differentiation Antigens on Normal Thymocytes.

The major work of this project was designed to elucidate and examine potential thymocyte subpopulations on the basis of cell surface differentiation markers.

It has been assumed from cytotoxic data that normal thymocyte populations were homogeneous with respect to phenotypic cell surface expression of Lyt 123 antigens. Other work from a number of investigators had detected minor subpopulations in the thymus which appeared to be like more mature peripheral T cells in terms of functional capabilities. Furthermore recent work in our laboratory with a series of thymic lymphomas suggested the possibility of a minor population of thymocytes differentiated for Lyt phenotype.

Using immunofluorescence and flow microfluorometry for quantitative analysis of Lyt antigens, a subpopulation, representing 10% of normal thymocytes, was detected which was Lyt 1^+23^-. By dual parameter analysis for fluorescence and light scatter, it was determined that these cells were larger than the average population and expressed more Lyt 1 antigen per cell. The fraction of cells expressing this phenotype increased from 10% to 30-60% in animals 36-40 hours after cortisone acetate treatment in vivo.

While it has been assumed that all thymocytes are Lyt 123^+ and that some of these cells differentiate to Lyt 1^+23^- or Lyt 1^-23^+ in the periphery, the current data suggest that it is equally likely that there are two distinct pathways for thymocytes in the thymus. One pathway might be for Lyt 1^+23^- cells which do not express Lyt 23 antigens during the course of functional differentiation. The second pathway might be for cells which express Lyt 123 antigens, presumably the precursors for both killer and suppressor T cells in the periphery.

(B) Cell Surface Phenotype of Thymocytes Fractionated on the Basis of Physical or Chemical Properties.

Using several separation techniques on thymocytes; sedimentation velocity, bovine serum albumin (BSA) density gradient, and peanut lectin agglutination, we have been able to show that there are at least two subpopulations of thymocytes with different Lyt phenotypes.

The three techniques have shown that: 1) There is no selective enrichment for Lyt 1^+23^- cells in the sedimentation velocity, although a clear separation on the basis of size is detected by light scatter and Coulter volume measurements. This analysis yielded data that indicate that light scatter is not directly correlated with cell size but rather is apparently correlated with nuclear to cytoplasmic ratio.

2) BSA density separations clearly enrich for Lyt 1^+ bright, Lyt 23^- cells in the lowest density fractions whereas the highest density fractions

are dull Lyt 1^+ Lyt 23^+.

3) Agglutination with peanut lectin strongly enriches for Lyt 123^+ cells in the agglutinated fraction versus Lyt 1^+23^- cells in the non-agglutinated fraction. From recent data of the other workers this fractionation appears to be on the basis of free galactose versus sialic acid as a terminal residue found on some cell surface glycoprotein. We have determined that the separation is not dependent on the Lyt 23 antigens because the separation works for both Lyt 2 allelic specificities as well as Lyt 3.2 and pretreatment of thymocytes with anti-Lyt 2 does not inhibit peanut lectin agglutination. The separation is not complete and it is possible that the nonagglutinated fraction contains two separable Lyt phenotypes, Lyt 1^+23^+ as well as Lyt 1^+23^-.

We are currently attempting to determine whether these non-agglutinated cells represent functionally differentiated thymic subpopulations which correlate with data on Lyt differentiated peripheral T cells.

II. Genetic Analysis of Recombinant Inbred Strains of Mice for Thymus Differentiation Markers.

In association with Dr. R. Riblet, et al., at the Cancer Research Institute, Philadelphia, PA, Lyt 2 typing has been conducted on a series of NZB x C58 recombinant inbred (RI) strains of mice. The strains were tested at F 16-21 for Lyt 2.1 or Lyt 2.2 and associated kappa immunoglobulin light chain markers. This study confirmed that Lyt 2 is tightly linked to a gene controlling V_k structure, since no crossovers were detected between Lyt 2 and Vk-PC8 or Vk-IF. These latter two markers are for particular patterns of light chain banding in isoelectric focusing gels. The first marker is observed after immunization for phosphorylcholine (PC) and is also associated with the light chain of a PC binding myeloma. There were a number of heterozygotes detected at F 16-21, where we would have expected no more than one, which raised the possibility that there is some selective advantage for heterozygosity in this genetic region. Alternatively there may be a system of forced heterozygosity linked with these gene loci.

Publications:

Mathieson, B. J., Campbell, P. S., Potter, M., and Asofsky, R.: Expression of Ly 1, Ly 2, Thy 1 and TL differentiation antigens on mouse T cell tumors. J. Exp. Med. 147: 1267-1279, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00137-12 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biology of GVH Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Asofsky	Laboratory Chief	LMI/NIAID
OTHER:	Colette Kanellopoulos-Langevin	Visiting Fellow	LMI/NIAID
	Herbert C. Morse III	Senior Investigator	LMI/NIAID

COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Little progress was made in the last half of the year because BALB/cN production was stopped and this is an essential strain. Work done in the first half of the year: See Z01 AI 00176-01 LMI.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00138-04 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Viruses and the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herbert C. Morse III	Senior Investigator	LMI/NIAID
Other:	R. Asofsky	Laboratory Chief	LMI/NIAID
	B. Mathieson	Staff Fellow	LMI/NIAID
	T. Chused	Senior Investigator	LMI/NIDR
	J. Hartley	Senior Investigator	LVD/NIAID
	W. Rowe	Laboratory Chief	LVD/NIAID
	S. Sharrow	Biologist	IB/NCI
	J. Goode	Biologist	LMI/NIAID
	B. Taylor*		
	R. Graff**		
	E. Stockert***		

COOPERATING UNITS (if any)
*The Jackson Laboratory, Bar Harbor, Maine
**Washington University, St. Louis, Missouri
***Memorial Sloan Kettering Institute, New York, New York

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Experimental Pathology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7/12	7/12	0/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An antigen related to the major glycoprotein of xenotropic murine leukemia viruses, termed XenCSA, was shown to be a normal constituent of the cell surface of mouse lymphocytes. The level of antigen expressed on lymphocytes of different inbred strains varies considerably and appears to be regulated by several genes. In crosses between C57BL/6 and DBA/2 mice, the major regulatory gene is at or near the Fv-1 locus on chromosome 4. In crosses between DBA/2 and C57L, the influence appears to be determined by a gene on chromosome 1. Studies involving H-1 congenics suggest that another gene on chromosome 7 may influence XenCSA expression.

Adult mice infected with rabies virus were shown to experience increased mortality when their humoral immune response was suppressed by treatment with antibody directed against IgM. The B cell response thus appears to be important in clearing rabies virus from the neuroparenchyma.

Project Description:

Murine Leukemia Virus

Rabbit antisera have been prepared which recognize the set of gp70 molecules produced by xenotropic but not ecotropic or amphotropic murine leukemia viruses (MuLV) and antigens termed XenCSA present on the surface of lymphocytes from all inbred strains of mice. Inbred strains vary markedly in the amount of antigen expressed on lymphocytes and these differences in expression are under genetic control. The genes responsible for regulation of antigen expression have been investigated in several crosses between strains expressing high and low levels of antigen.

(1) DBA/2 (high) by C57BL/6 (low). Studies of recombinant inbred strains generated by crosses between these strains and backcrosses of (C57BL/6 x DBA/2)F_1 mice to the parental strains have shown that Fv-1 or a closely linked gene on chromosome 4 regulates the level of virus expression; Fv-1^n from DBA/2 is associated with high expression and Fv-1^b from C57BL/6 with low expression.

(2) DBA/2 (high) by C57L (low). This strain combination is compatible at Fv-1, both bine Fv-1^n. In this cross, backcross studies indicate that a gene on chromosome 1 around 30 map units from 1n determines the level of XenCSA expression.

(3) B10.CE(62 NX)-H-$1^?$ (high) by C57BL/10 (low). Previous work indicated that there was excellent correlation between the XenCSA phenotype of inbred strains and the H-1 congenics derived from these strains on a B10 background. To determine if a gene determining high XenCSA expression is linked to H-1, we initiated studies of B10.CE(62NX)-H-$1^?$ mice which were segregated from high and low expression of XenCSA. High and low lines of this strain have been established which appear to differ by only a semidominant gene regulating XenCSA expression. An in vitro cell mediated cytotoxicity assay system has been developed which distinguishes between the H-1 alleles of B10 and B10.CE(62NX). Backcross mice [B10.CE(62NX) x B10]F_1 x B10.CE(62NX) will be studied to determine if the gene regulating XenCSA expression is linked to H-1.

(4) NZB (high) by NFS (low). NZB mice have 2 loci which determine expression of infectious Xenotropic MuLV. Congenic lines on an NFS background are being made for both of these independently segregating genes. These lines have been carried to backcross generation 5 and for both, high XenCSA expression is associated with expression of infectious virus. A third line, now in backcross generation 4 appears to express high levels of XenCSA in the absence of virus production.

RABIES VIRUS

Adult mice infected with the HEP strain of rabies develop a subclinical infection and clear virus completely from their brains. Mice depleted of B

cells by treatment from birth with anti IgM antibody develop a lethal infection with persistance of rabies virus in the brain. These results demonstrate that B cells or their products play a major role in clearing the neuroparenchyma of rabies virus.

Publications:

Miller, A., Morse, H. C. III, Winkelstein, J., and Nathanson, N.: The role of antibody in recovery from experimental rabies. I. Effect of depletion of B and T cells. J. Immunol, in press.

Chused, T., and Morse, H. C. III: Expression of XenCSA, a cell surface antigen related to the gp70 of Xenotropic murine leukemia viruses, on lymphocytes of inbred mice. In, Origins of Inbred Mice. (ed. H. C. Morse III), Academic Press, New York, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00139-13 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Immunologic Response of Animals to Trypanosomal Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Finerty	Research Microbiologist	LMI/NIAID
OTHER:	L. Kendrick	Biologist	LMI/NIAID
	L. P. Gasbarre*	Guest Worker	
	R. McKelvin**	Student	

COOPERATING UNITS (if any)

*Department of Zoology, University of Maryland
**Howard University

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12/12	6/12	6/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mice immunized with trypanosomal antigens demonstrated delayed hypersensitivity and protection. Only DH reactions were elicited with live trypomastigotes. Mice with little or no IgM responses survived three times longer when infected, than high IgM responders. This indicated an inhibitory role of IgM antibody in trypanosome infections.

Project Description:

The purpose of this study was to assay the host immune response in animals immunized or infected with African trypanosomes - the causative agent of African Sleeping Sickness. The parasites are lethal to laboratory animals, although death times vary with the parasite strain. Current studies focused on cell-mediated immune responses (CMI) and the role of IgM in the pathogenesis of African sleeping sickness. CMI responses were assayed by delayed-hypersensitivity reactions (DH) measured in the hind-footpad. Mice infected with various strains of Trypanosoma brucei or T-rhodesiense, showed no DH and succumbed to the infection. Other mice were immunized with trypanosomal antigens in order to induce DH. These mice were challenged with 10^4 live trypomastigotes and survived the challenge. A study of the kinetics of footpad swelling revealed an Arthus reaction at 3 hr. followed by a DH reaction at 24 hr. However, when live parasites were used as the eliciting antigen, only a DH reaction was observed. Athymic nude mice did not give DH and were not protected when challenged. These data indicated that a functional T-cell system was necessary for protection. Further, T-cells may function as cytotoxic or helper cells and these possibilities are currently under study.

High IgM levels are a characteristic response in mammals infected with trypanosomes. Normal mice and mice unable to synthesize IgM were infected with T. brucei and T. rhodesiense. IgM and parasite levels were assayed at appropriate times after infection and correlated with day of death. Mice that showed high IgM responses died 30 days after infection. Mice that showed little or a delayed IgM response, survived up to 100 days, a 3-fold increase in survival time. These data indicated that both IgM antibody and mitogen responses produced in response to trypanosomal antigens, played a role in the pathogenesis of the disease. The results suggested that IgM antibody is detrimental to the host, possibly forming antibody-antigen complexes.

An adjunct study of secretory immunoglobulins revealed that mice with little or no IgM responses to the human intestinal parasite Hymenolepis nana had high IgA and IgE levels. IgE was measured by passive-cutaneous anaphylaxis (PCA) in Fisher rats. Serum obtained from these mice showed greater protection when passively transferred to normal recipients. Future studies will assay IgE antibody and its role in protection against other parasites.

Publications:

Finerty, J. F., Krehl, E. P. and McKelvin, R. L.: Delayed-type hypersensitivity in mice immunized with Trypanosoma rhodesiense antigens. Inf. & Imm. 20: 464-467, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00140-13 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunology of Malaria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Finerty	Research Microbiologist	LMI/NIAID
OTHER:	L. Kendrick	Biologist	LMI/NIAID
	R. McKelvin	Student	LMI/NIAID
	W. P. Weidanz*	Professor	LMI/NIAID
	R. Rank**	Assistant Professor	LMI/NIAID
	D. Roberts***	Research Microbiologist	LMI/NIAID

COOPERATING UNITS (if any)

*The Hahneman Medical College, Phil., Pa.
**University of Arkansas for Medical Sciences, Little Rock, Arkansas
***National Toxicologic Research Center, Little Rock, Arkansas

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Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section

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NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12/12	6/12	6/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mice immunized with malarial antigens demonstrated delayed hypersensitivity to malarial antigens. Swiss mice revealed greater DH than the inbred strains assayed, and this correlated with the greater protection seen in Swiss mice to malarial parasites.

Project Description:

It is the purpose of these studies to assess the nature of the host immune responses to the rodent malarial parasites Plasmodium berghei, P. vinckei and two strains of P. yoelii - lethal and non-lethal strains. P. yoelii remains non-lethal when passaged in non-inbred Swiss mice. If transferred to inbred mice e.g. BALB/c mice, the non-lethal rapidly becomes lethal, suggesting that some strains of inbred mice allow the lethal population to come through. Another indication, is that "resident" viruses within a mouse colony also contribute to the death of infected mice. Analysis of delayed-hypersensitivity (DH) reactions, enhanced by cyclophosphamide (Cy) treatment indicated that NIH Swiss mice produce greater DH reactions than other inbred strains. This suggests that Swiss mice have a more active T-cell population and/or a greater T-cell repetoire capable of responding to the lethal P. yoelii population under appropriate conditions. Finally, DH was induced to P. vinckei parasites, a more lethal malarial parasite than either P. berghei or lethal P. yoelii. Future studies will focus on the degree of cross-reactivity among P. berghei, P. yoelii and P. vinckei in DH reactions. This will allow an analysis of the cell-mediated immune responses, particularly T-cell-macrophage interactions and the role of T-helper cells, in protection against these parasites.

Publications:

McMaster, P. R. B., Owens, J. D., Prescott, B., Finerty, J. and Weichbrod, R.: The induction of immunologic tolerance with Type III pneumococcal polysaccharide cross-linked or coupled to protein. Ann. Immunol. (Inst. Pasteur), 129C: 337-345, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00141-04 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immune Response to Malaria and Malarial Antigen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Asofsky	Laboratory Chief	LMI/NIAID
OTHER:	Y. Rosenberg	Visiting Fellow	LMI/NIAID
	C. Evans	Biological Lab Technician	LMI/NIAID

COOPERATING UNITS (if any)

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Laboratory of Microbial Immunity

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Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland

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13/12	9/12	4/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mice given a nonlethal strain of Plasmodium berghei develop a substantial increase in immunoglobulin (Ig) formation as judged by reverse plaques. Most of these antibody forming cells were nonspecific, but a few made antibody to bromelain-treated mouse erythrocytes, i.e. was an autoantibody. The nonspecific increase occured in all Ig classes.

Project Description:

BALB/c mice were infected with Plasmodium berghei yoelii NL a nonlethal strain. Spleen cells from different times after infection were analyzed for production of IgG1, IgG2, IgA, and IgM by the method of reverse plaques. There were 100 fold increases in numbers of cells secreting Ig of all classes. An increase in antibodies to sheep red cells was sustained; that to bromelain treated mouse RBC peaked earlier, but then subsided. Irradiated parasites gave none of these increases except the one to bromelain-treated mouse cells, which showed identical kinetics with that seen in the active infection.

Publications:

Rosenberg, Yvonne J.: Autoimmune and polyclonal B cell responses during murine malaria. Nature 274:170-172, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00142-05 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Development of Thymus-derived (T) Suppressor and Amplifier Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. J. Baker — Head, Microbiology and Immunology Section, LMI/NIAID

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during current year.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00143-09 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Genetic Control of the Antibody Response to Type III Pneumococcal Polysaccharide (SSS-III).

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Research Microbiologist	LMI/NIAID
Other:	B. Prescott	Microbiologist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	P. W. Stashak	Microbiologist	LMI/NIAID

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
11/12	7/12	4/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Backcrosses and intercrosses between recombinant-inbred and progenitor strains of mice confirmed that bone marrow-derived precursors of antibody-forming cells (B cells), suppressor T cells, and amplifier T cells participating in the antibody response to Type III pneumococcal polysaccharide (SSS-III) are under multigenic control. The genes involved act in a complimentary manner and are not linked to the major (H-2) histocompatibility complex or to the IgC_H allotype complex. Congenic resistant strains of mice, selected for known genetic differences within the H-2 genetic complex, were found to differ significantly in their capacity to give an antibody response to SSS-III; this suggests that such strains may differ at other unselected genetic loci, including those involved in determining non-H-2-associated antibody responses to SSS-III and perhaps other antigens.

PHS-6040
(Rev. 10-76)

Project Description:

In previous studies conducted in this laboratory, recombinant-inbred (RI) strains of mice, as well as the progenitor strains from which they were derived, were evaluated with respect to the capacity of bone marrow-derived precursors of antibody-forming cells (B cells) to respond to an optimally immunogenic dose (0.5 μg) of Type III pneumococcal polysaccharide (SSS-III), in addition to the amount of thymus-derived (T) suppressor and amplifier activity present. Several autosomal dominant genes, acting in an independent manner, were found to influence the expression of each of these functions which determine the magnitude of the antibody response produced after immunization. None of these functional activities was found to be linked to genes within the major histocompatibility (H-2) or the IgC_H allotype complex.

Preliminary studies showed that progeny derived from crosses between low and intermediate responding RI strains of mice gave antibody responses, 7-20 times greater than those of either parental strain used. Consequently, additional evidence for such genetic complementation was sought in other backcross combinations with respect to both B cell and amplifier T cell functions; this information would provide a better estimate of the number of genes actually involved in the expression of these activities. Using this experimental approach, the following observations were made with respect to B cell function. First, progeny derived from crosses between some low responding RI strains gave antibody responses that were significantly (3-6 times) greater than that of either parental strain used in the cross; this confirmed the fact that genes governing the capacity of B cells to give an antibody response to SSS-III indeed act in a complementary (additive) manner. Second, some RI strains of mice give identical antibody responses to SSS-III; but, when these strains were backcrossed to the same low responding strain, the progeny derived from such backcrosses did not give similar responses to SSS-III. This means that (a) strains of mice which give similar antibody responses to SSS-III may not necessarily share the same genotype, and that (b) based on the results of these and previous studies, as many as 5 genes could influence the capacity of B cells to give an antibody response to SSS-III.

Complementary effects were also noted with respect to the expression of amplifier T cell activity in progeny derived from different backcrosses, and intercrosses. However, in some cases, these progeny had amplifier T cell activity, significantly below that of either parental strain used; this could be due to incomplete penetrance, or to differences between parental strains in the amount of suppressor T cell activity present. Suppressor T cells are known to act on both B cells and amplifier T cells to limit the magnitude of the antibody response and other studies have demonstrated an inverse relationship between suppressor and amplifier T cell activity.

Six different strains of congenic resistant (B10) mice, which are known to differ at several loci within the major (H-2) histocompatibility complex, were assessed for their capacity to make an antibody response to an optimally

immunogenic dose of SSS-III; these strains were found to differ significantly (as much as 13-fold) in the magnitude of the antibody response produced. Other studies conducted with SSS-III have established that genes governing responsiveness to this antigen are not associated with the major (H-2) histocompatibility complex. However, since the congenic resistant strains used were bred and selected only for genetic differences within the H-2 complex, they could conceivably differ at other unselected loci, including those involved in determining responsiveness to SSS-III and perhaps other antigens. The existence of multiple genes influencing both B cell and regulatory T cell function, in conjunction with the backcross procedure used would allow the genes involved to segregate - and eventually to become fixed - in the congenic resistant strains in question, thereby resulting in significant differences in responsiveness to SSS-III. The basis for such differences is now being explored.

Publications:

Baker, P. J., Amsbaugh, D. F., Prescott, B., Stashak, P. W., and Rudbach, J. A.: Multigenic control of the antibody response to Type III pneumococcal polysaccharide and other helper T cell independent antigens. In, Friedman, H., T. J. Linna, and J. E. Prier (eds), Infection, Immunity, and Genetics. University Park Press, Baltimore, Md., in press.

Pasanen, V. J., Asofsky, R. A., Prescott, B., and Baker, P. J.: The effect of allogeneic stimulation on the antibody response to Type III pneumococcal polysaccharide. Synthesis of two classes of antibody, γM and γG or γM and γA, by identical cells. J. Exp. Med., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00144-14 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Regulation of the Antibody Response to Type III Pneumococcal Polysaccharide (SSS-III).

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Research Microbiologist	LMI/NIAID
Other:	R. B. Markham	Research Associate	LMI/NIAID
	B. Prescott	Microbiologist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	P. W. Stashak	Microbiologist	LMI/NIAID
	N. D. Reed*		

COOPERATING UNITS (if any)

*Department of Microbiology, Montana State University, Bozeman, Montana

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Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12/12	8/12	4/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effect of a single dose of hydrocortisone on three different immunoregulatory functions influencing the magnitude of an antibody response of mice to Type III pneumococcal polysaccharide (SSS-III) was evaluated in the present work; these functions included suppressor, amplifier, and helper activity which are dependent upon the presence of distinct subpopulations of thymus-derived (T) lymphocytes. The results obtained show that a single injection of a relatively large dose of hydrocortisone eliminated both the generation and expression of helper T cell activity. But, under the same experimental conditions, suppressor T cell and amplifier T cell activities were uneffected, even in this steroid-sensitive species; hydrocortisone did not influence the capacity of bone marrow-derived precursors of antibody-forming cells (B cells) to respond to SSS-III. Such selective sensitivity to steroid may account for some of the immunosuppressive potency of steroids.

PHS-6040
(Rev. 10-76)

Project Description:

As knowledge of the mechanisms by which immune responses are generated has increased, the understanding of how adrenal glucocorticosteroids act to abrogate such responses has become more sophisticated. Massive lysis of lymphocytes occurs after steroid treatment; but such lysis is not demonstrable in all animal species and different mechanisms have been involved to account for the immunosuppression noted in "steroid-resistant" species. Lymphocytes consist of subpopulations of both thymus-derived (T) and bone marrow-derived (B) lymphocytes. Although T cells generally were found to be more steroid-sensitive than B cells, opinion has been divided on this issue. Furthermore, it has been suggested that in certain types of immune reactions, it is not the lymphocyte but the macrophage - an accessory cell needed for the development of an immune response to some antigens - which is critically sensitive to steroid.

Since T lymphocytes are now classified in terms of functional subpopulations capable of suppressing, helping or amplifying immune responses to various antigens, it is important to examine the effect of steroids on the expression of each of these functional activities in vivo in order to establish which subpopulation of T lymphocytes are being effected by steroid treatment. The expression of these three types of T cell functions has been well-characterized with respect to the in vivo development of an antibody response to Type III pneumococcal polysaccharide (SSS-III) in mice. The antibody response of mice to this antigen does not require the presence of helper T cells; but, the results of other studies conducted in this laboratory have shown that the magnitude of the antibody response to SSS-III is influenced, nevertheless, by the activities of two functionally distinct types of regulatory T cells, termed amplifier and suppressor cells. Although amplifier T cells play no role in the early recognition of antigen and in the initiation of an antibody response to SSS-III, they act at a later stage of the immune response and cause antigen-stimulated B cells to proliferate further in response to antigen. Suppressor T cells act by inhibiting both the activity of amplifier T cells and the extent to which B cells proliferate after immunization.

The effect of helper T cells on the magnitude of the antibody response to SSS-III can be assessed by immunizing with a helper T cell-dependent form of the antigen. This consists of SSS-III, linked to an appropriate carrier (e.g., horse red blood cells or HRBC) to form a complex (SSS-III-HRBC); pretreatment or priming with a subimmunogenic dose of the carrier then permits mice to generate an antibody response upon immunization with amounts of SSS-III-HRBC that would otherwise not be immunogenic in unprimed mice. Such an antibody response has been found to be both specific for SSS-III and dependent upon the generation of carrier-specific helper T cells, generated as a result of carrier priming.

We have examined the in vivo sensitivity to hydrocortisone succinate of suppressor, amplifier and helper T cell activity with reference to the antibody response of mice to SSS-III. The results obtained show that both the

generation and expression of helper T cell activity is eliminated or substantially reduced by treatment with a single dose of hydrocortisone; therefore, helper T cell activity is steroid sensitive. By contrast, neither amplifier T cell activity nor suppressor T cell activity is altered in mice given the same large dose of hydrocortisone; treatment with this dose of steroid did not influence the ability of B cells to respond to SSS-III. These findings suggest that the immunosuppressive effects of steroid may be due both to the removal of helper T cell activity needed for the initiation of antibody responses to some antigens and the sparing of suppressor T cells, which normally act to limit the magnitude of the antibody response produced after immunization.

Publications:

Markham, R. B., Stashak, P. W., Prescott, B., Amsbaugh, D. F., and Baker, P. J.: Generation of low-dose paralysis in the absence of the ability to secrete antibody. J. Immunol. 120: 986-990, 1978.

Markham, R. B., Stashak, P. W., Prescott, B., Amsbaugh, D. F., and Baker, P. J.: Sensitivity of amplifier T cells involved in the antibody response to Type III pneumococcal polysaccharide to antilymphocyte serum. J. Immunol. 119: 1159-1162, 1978.

Markham, R. B., Stashak, P. W., Prescott, B., Amsbaugh, D. F., and Baker, P. J.: Selective sensitivity to hydrocortisone of regulatory functions which determine the magnitude of the antibody response to Type III pneumococcal polysaccharide. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00145-11 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mode of Action of Thymus-derived (T) Suppressor and Amplifier Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Microbiologist	LMI/NIAID
Other:	K. B. Schroer	Research Associate	LMI/NIAID
	B. Prescott	Microbiologist	LMI/NIAID
	P. W. Stashak	Microbiologist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	K. J. Kim	Fogarty Fellow	LMI/NIAID

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
16/12	12/12	4/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were conducted to examine the specificity of the effects produced by amplifier T cells and suppressor T cells involved in the antibody response to Type III pneumococcal polysaccharide (SSS-III). The results obtained showed that (a) amplifier T cells generated after immunization with SSS-III influence only those antibody-forming cells stimulated by SSS-III, and that (b) the effects produced by suppressor T cells were antigen-specific with regard to both their induction and expression. The role of the idiotypic determinant of cell-associated antibody specific for SSS-III in the generation of these types of regulatory T cell activities is being assessed.

PHS-6040
(Rev. 10-76)

Project Description:

Although the results of previous studies conducted in this laboratory have provided much information concerning the mode of action of regulatory amplifier and suppressor thymus-derived (T) cells, the recognition units involved in promoting the effects produced remain to be identified. Thus, a more detailed examination of the specificity of amplifier and suppressor T cell activity would enable one to make some definite statements in this regard and to construct working hypotheses that can be tested experimentally. Treatment with antilymphocyte serum, at the time of immunization with Type III pneumococcal polysaccharide (SSS-III), results in a 10 to 20-fold increase in the magnitude of the antibody response produced; such ALS-induced enhancement has been attributed to the action of amplifier T cells, whose activities can be expressed after the removal of ALS-sensitive suppressor T cells. However, treatment of BALB/c mice with the same preparation of ALS does not increase the antibody response to α1,3 dextran. It therefore was of interest to determine whether ALS would increase the magnitude of the antibody response to <u>both</u> antigens given together at the time of ALS treatment; this did not occur. Instead, treatment with ALS resulted in a 15-fold increase in the antibody response to SSS-III, whereas the antibody response to α1,3 dextran remain essentially unchanged. In another study, mice were given ALS at the time of immunization with the conjugate TNP_7-SSS-III and the magnitude of the antibody response to both SSS-III and the trinitrophenyl (TNP) determinant was assessed at peak, 5 days later. Enhancement (about 10-fold) was evident only with respect to the antibofy response to SSS-III; the antibody response to TNP was unchanged. These data argue against the possibility that ALS-induced enhancement is due simply to splenomegaly or to a non-specific mitogenic effect of ALS on bone marrow-derived precursors of antibody-forming cells (B cells). More important, they suggest that the amplifier T cell activity generated during the course of an antibody response to SSS-III influences only those B cells stimulated by SSS-III.

Pretreatment (priming) with a marginally immunogenic dose (0.005 μg) of SSS-III greatly reduces the magnitude of an antibody response to an optimally immunogenic dose (0.5 μg) of SSS-III given 3 days - 2½ months later; such unresponsiveness (low-dose paralysis) has been shown to be an antigen-specific, T cell dependent phenomenon that can be induced under conditions in which priming does not result in the formation of detectable antibody. The specificity of low-dose paralysis, which is mediated by suppressor T cells, was examined by immunizing <u>low-dose primed</u> mice with an optimally immunogenic dose of SSS-III and another unrelated antigen (sheep erythrocytes, α1,3 dextran, polyvinylpyrollidone, or bacterial lipopolysaccharide); then, the magnitude of the antibody response to <u>both</u> antigens was measured at peak, 5 days later. In all cases, priming with a low dose of SSS-III resulted in significant (>80%) suppression of the antibody response <u>only</u> to SSS-III; antibody responses to the remaining antigens used were unaffected. In another study, mice were primed with a low dose (0.005 μg) of SSS-III; three days later, they were given an optimally immunogenic dose of TNP_7-SSS-III and the magnitude of the antibody response to both TNP and SSS-III was assessed at peak, 5 days later. Although significant suppression (>80%) was noted in

the case of the antibody response to SSS-III, the antibody response to TNP was unchanged as a result of priming. These studies illustrate a high degree of antigen specificity with regard to both the induction and expression of low-dose paralysis, i.e., suppressor T cell activity.

Although the effects produced by suppressor T cells, and perhaps amplifier T cells as well, appear to be antigen-specific, there is no evidence that T cells can recognize and respond directly to SSS-III. Consequently, we have proposed that the specificity observed may be the result of the ability of regulatory T cells to recognize the idiotypic determinant of cell-associated antibody specific for SSS-III. In order to test this hypothesis, it would be necessary to prepare quantities of anti-idiotype antibody; but, since the serum antibody response to SSS-III in mice is usually quite low (about 10-20 μg/ml), sufficient amounts of purified antibody are difficult to obtain for this purpose. We have recently begun cell fusion studies to accomplish this objective. Here, spleen cells from mice immunized with SSS-III are fused, in the presence of polyethyleneglycol, with plasma cells derived from a plasma cell tumor line (MPC 21). In one experiment the fused products were divided among 24 wells of which 30% were found to be making antibody specific for SSS-III. Most of these fusion products have been transferred sucessfully to mice; tumors developed and about 0.2-5 mg/ml of precipitating antibody specific for SSS-III was detected in the ascitic fluid. Such concentrations of antibody now make it possible for us to prepare anti-idiotypic antiserum which will be used to obtain information on (a) the role of the idiotypic determinant in the activation of regulatory T cells, and (b) the clonal diversity of cells making antibody specific for SSS-III in the genetic studies described in Z01-AI-00143-09, LMI.

Publications:

Markham, R. B., Reed, N. D., Stashak, P. W., Prescott, B., Amsbaugh, D. F., and Baker, P. J.: Effect of Concanavalin A on lymphocyte interactions involved in the antibody response to Type III pneumococcal polysaccharide. II. Ability of suppressor T cells to act on both B cells and amplifier T cells to limit the magnitude of the antibody response. J. Immunol, 119: 1163-1168.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00146-05 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Chemical, Biochemical and Immunological Studies on Biologically Active Fractions Isolated from Various Plant, Bacterial and Parasitic Sources.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Prescott	Head, Biochemistry Section	LMI/NIAID
	P. J. Baker	Head, Microbiology & Immunology Section	LMI/NIAID
	J. F. Finerty	Research Microbiologist	LMI/NIAID
Other:	G. Caldes	Chemist	LMI/NIAID
	C. Thomas II*	Research Plant Pathologist	
	H. Cathy**	Laboratory Chief	
	P. Z. Allen***	Professor	
	P. R. Beining****	Guest Worker	LMI/NIAID
	C. Thomas III	Bio Aid (Biochemistry)	LMI/NIAID
	K. Schroer	Research Associate	LMI/NIAID
	G. Flannery	Biologist	LMI/NIAID

COOPERATING UNITS (if any) *Applied Plant Pathology Lab., USDA, Agricultural Research Service, Beltsville, Md. **Ornamentals Laboratory, USDA, Beltsville, Md. ***Univ. of Rochester School of Medicine & Dentistry, Rochester, N.Y. ****Univ. of Scranton, Scranton, PA.

LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Biochemistry Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
22/12	10/12	12/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were continued on the isolation, purification and characterization of biologically active polysaccharides from pneumococci types 6, 9, 10, 21, 22, 29, and 67, Streptococci A, B, F, MG and faecium, Lactobacillus bifidus, mirococcus lysodeikticus, Hydrogemonas eutrophia, Hydrogemonas, Bacillus subtilis, and polysaccharides from 10 additional ornamental flowers. The polysacchardies were tested for cross reactivity with Type III pneumococcal rabbit and horse antisera. Forsythia and magnolia polysaccharides reacted with low titers to horse anti-serum to type III pneumococcus in precipitin tests. Several oligosaccharides from B 512 dextran and type 6 pneumococcus polysaccharide were prepared in addition to complexes of stearoyl, octadecylisocyanate, octadecyliosothiocyanate, and tricarbanilate of B 512 dextran for immunological hapten inhibition in vivo studies. The properties and reactions of the cross reactive polysaccharides may be of considerable interest in the possible role of cross-reactive antigens on immunological reactions.

PHS-6040
(Rev. 10-76)

Project Description:

Lipids:

Lipid studies were continued on the flowers of ornamental plants. Glycolipids isolated from these plants were analyzed for their carbohydrate content. The carbohydrates detected were glucose, galactose and small amounts of mannose. In previous reports, we have shown that glycolipids isolated from several plants possessed cross-reactivity with mycoplasma pneumoniae and Streptococci groups A and D. A continuation of these studies on cross reactive or shared determinants with these and other organisms (pneumococci) is being studied with the glycolipids of 30 ornamental plants. The methods used for the isolation and purification of the serologically reactive plant glycolipids have been described in previous reports. Glycolipids of DAUCUS CAROTA (wild carrot), Carthamus tinctorius (safflower) Lawsonia Inermis (henna) and others cross reacted in the complement-fixation test with rabbit antisera to m. pneumomiae and Streptococci group A and D. These findings show that certain plants and m. pneumoniae and Streptococci groups A and D share similar lipid antigens.

The glycolipids of Staphylococcus aureus 6538P and M. pneumoniae were tested for their effect on the immunogenicity of Type III pneumococcal polysaccharide (SSS-III). These glycolipids did not affect the immune response of BALB/c mice to Type III SSS.

Polysaccharides:

Studies were continued with Dr. C. A. Thomas II, Plant Pathologist of the U.S. Department of Agriculture, Experimental Station in Beltsville, Md. on the Carthamus tinctorius polysaccharide (CT). Additional large batches of the plant were grown by Dr. Thomas for out studies on the structure of the polysaccharide. The cross reactivity of this polysaccharide with type III pneumococcal polysaccharide was described in last year's report. The antigenic activity of the CT polysaccharide in Ouchterlony tests with pneumococcus type III horse antiserum was found to be stable when the polysaccharide was heated at 80°C for 20 hours. Hydrolysis of the CT polysaccharide in 0.1 N H_2So_4 for 4 hours removed 10% fructose, 3% arabinose, and 0.5% galactose but did not destroy the precipitability with type III rabbit and horse antisera.

Treatment of the CT polysaccharide with B-glucuronidase destroyed the precipitability with the type III horse antiserum. This enzyme also destroyed the activity of SSS-III with horse antiserum. Fractionation of the CT polysaccharide with 0.5, 1, 2 and 4 volumes of ethanol successively yielded five fractions including the final alcohol supernatant, all of which were active in the Ouchterlony tests with Type III horse antiserum. Sixty percent of the active material was present in the 2 volume alcohol precipitate. Thus, it seems that the active material must possess a certain molecular configuration for antigenic activity, since the size of the molecule did not affect the antigenic activity of the polysaccharide.

Methylation studies on the CT polysaccharide are being carried out to isolate methylated derivatives of the hydrolyzed CT polysacchairde by column chromatography and determine configuration of the molecule.

In addition to the above, polysaccharides from various bacteria (in summary) are being isolated as potential antiviral agents against plant viruses. The polysaccharide isolated from Bacillus subtilis was shown to inhibit the sugar cane mosaic virus.

Ornamental Plant Polysaccharides:

Since the polysaccharide isolated from the geranium flower cross reacted with type III pneumococcal horse antiserum the relationship between the geranium polysaccharide and the type III pneumococcal polysaccharide became clearer with the demonstration that the geranium polysaccharide is a polymer which contains galactosyl-glucosyl-uronic acid units joined together in β-1, 3 linkages. Hydrolysis of the polysaccharide show the majority of the β-1,3 linkages occur in linear chain position and that the glucose appears to be contiguous with uronic acids.

Pneumococcal Polysaccharides:

In continuation of our studies with Dr. Peter Z. Allen, Professor of Immunochemistry at the University of Rochester Medical School on the cross reactivity and immunochemistry of Mycoplasma pneumoniae polysaccharide and type 23 and 32 pneumococcal polysaccharides; cross-precipitation in type specific rabbit antipneumococcal anti-type 23 and anti-type 32 sera cross was examined in detail and could be attributed to a rhamnose-galactose rich component of M. pneumoniae polysaccharide fraction recovered from immunoprecipitates formed with anti-type 23 serum. Immunochemically isolated mycoplasma polysaccharide was found to contain glucose, galactose, rhamnose and mannose in 1:14:5:4 molar proportions. Comparison of the ability of 6-O-α-L-rhamnosyl-D-glucose and free rhamnose to inhibit precipitation by homologous pneumococcal and heterologous mycoplasma antigens indicates a combining site specificity for anti-type 23 and anti-type 32 antibodies directed largely against the α-linked L-rhamnosyl determinants and the occurrence of α-L-rhamnosyl units in type 32 and M. pneumoniae polysaccharides. Hapten inhibition of the cross-precipitation of pneumococcal type 23 capsular polysaccharide in anti-type 32 serum helps to establish that cross-reactivity can be attributed to interaction of α-L-rhamnosyl units of type 23 with anti α-L-rhamnoside combining sites of anti-type 32 antibodies.

Polysaccharides from pneumococci types 21, 22, 29 and 67; Streptococcus group and type polysacchardie antigens from group A, D, F; Streptococcus mutans and Streptococcus MG, were isolated from cultures grown in Todd-Hewitt medium for additional studies on cross-reactivity with M. pneumoniae and with Streptococcus MG. Type III pneumococcal polysaccharide is prepared regularly for continued genetic and immunological studies with Dr. P. J. Baker.

In last years report, we described the isolation of polysaccharides

from the malarial parasites Plasmodium berghei and Plasmodium vinckei. Preliminary studies showed that the polysaccharide from P. berghei contained 0.9% N and 0.4% P while the polysaccharide isolated from P.vinckei was slightly higher 1.2% N and 0.8% P. Chromotography of acid hydrolysates of the polysaccharides in ethyl acetate-pyridine-water and aniline malonate reaction revealed the presence of the following carbohydrate hexoses-glucose, galactose and mannose and the pentose xylose. Studies are in progress to determine whether these polysacchardies cross react with the type III pneumococcal polysaccharide and with the Mycoplasma pneumoniae polysaccharide since they contain similar antigenic carbohydrate determinants.

Chemical Synthesis:

(1) Oligosaccharides of B 512 dextran were prepared by hydrolyzing 50 grams of material with 6NHCl for 4 hours, neutralized and dialyzed. The dialyzable oligosaccharides were concentrated, taken up in one percent aqueous ethanol and poured onto a column containing 200 grams of charcoal and 100 grams of Celite. The column was washed with one percent ethanol and the oligosaccharides eluted successively with 5, 7.5, 10, 12.5 and 15 percent ethanol. Two liter amounts were collected and the eluate concentrated by lyophilization. The samples were given to Dr. Ken Schroer for his studies on hapten inhibition.

(2) Tricarbanilate esters of B 512 dextran were synthesized by dissolving one gram of B 512 dextran in 100 ml pyridine, adding 4 grams of p-nitrophenyl isocyanate and the mixture stored for 3 days at room temperature. In several hours the mixture became very thick and insoluble. The mixture was precipitated with 5 volumes of 95% ethanol, filtered through a # 41 Whatman paper on a Buchner funnel and the precipitate washed well with ethanol and ether. Dried at room temperature. Analytical analysis showed the compound to be dextran tricarbanilate M. P.>300^{o}C.

(3) The esters of octadecylisocyanate and octadecylisothiocyanate were prepared like No. 2.

(4) The stearoyl ester of B 512 dextran was prepared as follows: 6.4g of B 512 dextran plus 4.2g phenylboronic acid were dissolved in 100ml dry dimethylsuloxide and 100ml benzene added gradually while heating to reflux. Water from the reaction was collected in a Dean-Stock apparatus for 18-24 hrs until no further increase. The reaction product was freed from benzene by evaporation. The product was dissolved in pyridine and stearoyl chloride 20mg/50mg of dextran added. The mixture was stirred over night and precipitated with 2-propanol, filtered, and the solution was dialyzed 24 hrs vs distilled water. The material was lyophilized and dried.

These compounds are being used for immunochemical studies.

The joint project with Dr. Paul R. Beining, Professor of Biology at the University of Scranton (Guest Worker, LMI) and Geraldine Flannery, graduate student, University of Scranton (Biologist, LMI) was continued with studies to determine the chemical nature of the protein present in the

Staphylococcus aureus 6538P lipoteichoic acid (LTA) molecule and whether the complete complex molecule of LTA is essential for its antigenic activity. If the LTA without protein is the active principle, then the protein may be tied up with the active component either as a carrier or as an inhibitor under physiological conditions so that conversion of the bound LTA protein to the free LTA is necessary for immunological activity. The protein was separated from the LTA by trichloracetic acid precipitation. The product obtained is a white amorphous powder, soluble in water, non-dialyzable and gives positive reactions with all the known protein tests. The composition and chemical and physical properties of this material is being determined. Hydrolysis of the protein with 6NHCl for 18 hrs at 120-130°C and chromatographing the hydrolysate showed the following eleven amino acids: alanine, arginine, aspartic, glutamic, glycine, isoleucine, leucine, lysine, serine, threonine, and valine. Immunological and serological studies are being carried out with this material.

Pneumococcal types 6, 9, 10 and 29 polysaccharide were given to Dr. Beining for his studies on the cross reactivity of these polysaccharides with the LTA since these polysaccharides contain a ribitol phosphate molecule in their structure similar to that found in the LTA of S.aureus 6538P.

Special services were performed for a member of Laboratories at the NIH and other government and University laboratories.

C-pneumococcal substance was prepared from Type III pneumococci by acid extraction and column chromatography. Purified material was given to Dr. Kim Bottomly for her studies on coupling C-substance to sheep red blood cells as an indicator for anti PC ab response.

Type III pneumococcal polysaccharide (SSS-III) was sent to Dr. Robert Zuri, University of Conn. Health Center, Farmington, Conn., for his studies on arthritis.

Type III pneumococcal polysaccharide to Dr. C. Glaudemans, LC/NIAID for his studies.

Type III pneumococcal polysaccharide to Dr. Arthur Friedlander, Univ. of California, 225 West Dickinson St., San Diego, CA, for his experimental studies with macrophages.

Advisor to Geraldine Flannery in her work for her Master of Science Degree in chemistry at the University of Scranton.

Purified some mycoplasma pneumoniae glycolipid by column chromatography for Lee Cline, LID/NIAID.

Type 8 pneumococcal polysaccharide isolated for Dr. Phil McMaster.

Projections:

An intensive study is in progress to isolate purify and determine the molecular configuration of additional serologically cross-reactive polysaccharides from plant materials and from some of the higher type pneumococcal polysaccharides (Types 23 and 32) since they cross react with Mycoplasma pneumoniae polysaccharides. Last year's projections are in progress. Similar studies are being carried out with pneumococcal types 21, 22, 29, and 67 and additional plant polysaccharides. Increased emphasis will be placed on these studies because of the availability of antigenic components for testing. Emphasis is being placed on the relationship of steric configuration of the polysaccharide molecule and the carbohydrate components involved in antigenic specificity. Partial hydrolysis of the polysaccharide molecule is very important in these structural studies. With particle breakdown products of the groups and components in the molecular configuration of the serologically active polysaccharides, the exact molecular weights and the overall shape of the molecule can be determined. The plant polysaccharides could be utilized for intracellular detection of antibodies and employed for the characterization of the antibodies after immunoelectrophoresis and may be useful in studies of active sites and preparation of modified and synthetic antigens by chemical means which would not affect the antigenic activity but increase the immunogenicity of the molecules. The immune responses elicited by such artificial antigens may be important in areas such as immunological tolerance, allergy and genetics.

Publications:

Allen, P. Z., and Prescott, B.: Immunochemical studies on a Mycoplasma Pneumoniae polysaccharide fraction: cross reactions with types 23 and 32 antipneumococcal rabbit sera. Infection and Immunity 20: 421-429, 1978.

McMaster, P. R. B., Owens, J. D., Prescott, B., Finerty, J., and Weichbrod, R.: The induction of immunologic tolerance with Type III pneumococcal polysaccharide cross-linked or coupled to protein. Ann. Immunol. (Inst. Pasteur) 129C: 337-345, 1978.

Markham, R. B., Reed, N. D., Stashak, P. W., Prescott, B., Amsbaugh, D. F., and Baker, P. J.: Effect of Concanavalin A on lymphocyte interactions involved in the antibody response to Type III pneumococcal polysaccharide. II. Ability of suppressor T cells to act on both B cells and amplifier T cells to limit the magnitude of the antibody response. J. Immunol. 119: 1163-1168, 1977.

Markham, R. B., Stashak, P. W., Prescott, B., Amsbaugh, D. F., and Baker, P. J.: Generation of low-dose paralysis in the absence of the ability to secrete antibody. J. Immunol. 120: 986-990, 1978.

Markham, R. B., Stashak, P. W., Prescott, B., Amsbaugh, D. F., and Baker, P. J.: Sensitivity of amplifier T cells included in the antibody response to Type III pneumococcal polysaccharide to antilymphocyte serum. J. Immunol. 119: 1159-1162, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00153-02 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

In Vitro Responses of Human Peripheral Lymphocytes to Infections Organisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Asofsky	Laboratory Chief	LMI/NIAID
OTHER:	Colette Kanellopoulos-Langevin	Visiting Fellow	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0/12		

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

No progress was made this year since subjects sensitized to influenza A/Victoria could not be found.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00158-02 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Immune Response to Entamoeba Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. F. Finerty Research Microbiologist LMI/NIAID

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline)

Inactive during current year.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00176-01 LMI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Interrelation of Lymphoid, Musculo-skeletal, and Central Nervous System Organization

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Carol Lynn Ludwig	Research Associate	LMI/NIAID
OTHER:	Herbert C. Morse III	Senior Investigator	LMI/NIAID
	Susan Sharrow	Biologist	IB/NCI
	K. Jin Kim	Visiting Fellow	LMI/NIAID
	B. J. Mathieson	Staff Fellow	LMI/NIAID
	C. Kanellopoulos-Langevin	Visiting Fellow	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
16/12	16/12	0/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline !

The identification of cell surface antigens shared by components of the lymphoid, musculo-skeletal, and/or central nervous systems offers the possibility of (1) exploring multisystem membrane abnormalities through the investigation of lymphoid cells, easily obtained and purified; (2) defining subpopulations through the preparation of fluoresceinated reagents and subsequent analysis on the fluorescence activated cell sorter (FACS); and (3) clarifying the role of sub-populations in disease states affecting both the lymphoid and central nervous systems. These investigative areas are being pursued in projects exploring immunologic abnormalities in dystrophic mice, the distribution of G_{m1} ganglioside on lymphocytes, and the effects of neurotropic influenza A on the immune system.

PHS-6040
(Rev. 10-76)

Project Description:

Immunologic abnormalities in dystrophic mice: a possible association of neuromuscular and lymphoid defects was suggested recently when investigators described abnormalities of thymic development and morphology in 129/ReJ-dy dystrophic mice. We repeated and extended these studies, analyzing over 50 mice of varying ages with either the dy mutation or the allelomorphic dy^{2J} gene, using sex-matched +/+ or +/? littermates as controls. T cell function was assessed by mitogen stimulation with PHA and Con A, mixed leukocyte cultures, and graft-versus-host assays and B cell function was examined by mitogen stimulation with LPS. In addition, the distribution of 4 cell surface antigens (brain-associated theta, Lyt, mouse immunoglobulin, and XenCSA) was assessed by flow microfluorometry on 90 thymocyte and 64 spleen cell preparations. Our findings show that dystrophic and control mice are identical in most parameters of T and B cell function and cell surface antigenic expression. Occasional dystrophic mice had a population of thymocytes, somewhat larger than controls, with an abnormal distribution of brain-associated-theta consisting of two peaks of fluorescence containing approximately equal numbers of duller and brighter staining cells. These thymocyte populations also had an increased number of Lyt 2.2 negative cells. Inconsistently associated with these abnormalities of cell surface antigenic expression were an increased thymocyte proliferative response and a decreased spleen cell proliferative response to PHA.

The distribution of G_{m1} ganglioside on lymphocytes in normal and neurological mutant mice: it is generally accepted that cholera toxin binds selectively to the G_{m1} ganglioside on cell surfaces. We have prepared a fluoresceinated cholera toxin reagent for use with the FACS to delineate and analyze lymphocyte subpopulations. We have completed preliminary ontogeny sutides of cholera toxin expression on thymocytes and spleen cells from BALB/c mice 1-8 days of age and found a markedly decreased fluorescence intensity on large lympocytes from both thymus and spleen with increasing age. We are extending these studies in an investigation of cholera toxin expression on lymph node and bone marrow cells prior to studying, with this reagent, lymphocyte preparations from two strains of mutant mice with defects in myelin synthesis (quaking and $jimpy^{msd}$). The expression of cholera toxin is of special interest in these mice who have widespread white matter pathology since G_{m1} is one of the four major gangliosides in the brain. Possible associated abnormalities of immune function in these mice will be pursued in a manner similar to that described for the dystropic mice.

The effects of neurotropic influenza A on the immune system and subsequent central nervous system and thymic pathology: unpublished data indicate that viral antigen persists in the thymuses of mice infected with neurotropic influenza A (WSN strain). We plan to pursue this work in an effort to detail the interaction of virus with the immune system and the subsequent effects on pathology. We will repeat the experiment,

attempting viral isolation from acutely and chronically infected thymus and examining thymocytes for the presence of internal and cell surface viral antigens. We are in the process of preparing the following fluoresceinated reagents: anti-influenza A RNP, an internal antigen; anti-influenza A matrix protein, an internal antigen which is also expressed on the cell surface; and anti-hemagglutinin O, a cell surface antigen. We wish to assess whether lymphocyte subpopulations are preferentially infected by virus and to examine these effects. Mice depleted of T cell function, nude mice, and of B cell function, anti-μ suppressed mice, will be examined for their capability of withstanding viral infection and for the pathology produced under these conditions.

LABORATORY OF PARASITIC DISEASES

SUMMARY. 25-1

Z01 AI 00091-16 The Genetics, Biology, and Control of Snail Intermediate Hosts of Schistosomes - Richards 25-11

Z01 AI 00092-12 The pathogenesis of schistosome infections in mammalian hosts - Cheever 25-16

Z01 AI 00093-03 Studies of the Immunologic Response to Helminth Infections - Ottesen 25-19

Z01 AI 00094-19 Studies of Entamoeba histolytica and other Parasitic Protozoa - Diamond 25-23

Z01 AI 00097-20 Pathological Physiology and Histochemistry of Parasitic Diseases 25-27

Z01 AI 00098-22 Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms - Weinbach 25-30

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Z01 AI 00101-04 Characterization of Polysaccharides in protozoa of the family Trypanosomatidae - Gottlieb 25-39

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Z01 AI 00160-02 Studies on the Cell Biology of E. histolytica and other Species of Entamoeba - Gillin. 25-60

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Z01 AI 00162-02 Biochemical Cytology of Host-Parasite Interactions in Parasitic Protozoa - Dwyer 25-67

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Z01 AI 00105-05 Mechanism of Invasion of Erythrocytes by Malaria Merozoites - Miller 25-79

Z01 AI 00110-05 Gamete-Specific Immunity in Malaria - Gwadz 25-80

Z01 AI 00150-02 Enzyme Variation in Malaria and Babesia Parasites - Carter 25-81

Z01 AI 00159-01 Physiology and Culture of Sexual Stages of Malaria Parasites - Carter 25-82

Pacific Research Section, Honolulu, Hawaii

Z01 AI 00113-16 Studies on dengue - Rosen 25-83

Z01 AI 00115-04 Studies on the natural history of influenza virus in swine - Wallace 25-87

Z01 AI 00163-02 Field and laboratory studies on the transovarial transmission of Japanese and St. Louis encephalitis viruses by mosquitoes - Rosen 25-91

Z01 AI 00164-02 Epidemiology of Infectious Disease in the Pacific Region - Dean 25-94

Z01 AI 00177-01 Studies of the potential role of bedbugs as vectors of hepatitis B virus - Tesh 25-98

Z01 AI 00178-01 Studies of the mechanism of transovarial transmission of arboviruses by mosquitoes - Tesh 25-101

Terminated Projects, PRS, LPD, NIAID

Z01 AI 00114-06 Studies on the arboviruses of the Phlebotomus fever serogroup - Tesh 25-105

Z01 AI 00151-02 Studies on the ecology of encephalomyocarditis virus - Tesh 25-106

Z01 AI 00115-04 Studies on the natural history of influenza virus in swine - Wallace 25-87

Z01 AI 00164-02 Epidemiology of Infectious Disease in the Pacific Region - Dean 25-94

Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - October 1, 1977 through September 30, 1978

INTRODUCTION

The LPD continued to sponsor a variety of field research projects in various areas of the world. One team of Drs. Carter and Gwadz of the Malaria Section was joined by Dr. Green of LI for several months of work at the British MRC Laboratory in the Gambia, West Africa. Drs. Neva and Gusmao were joined by Mr. Poindexter in Brazil for special studies with Brazilian collaborators on Chagas' disease. Dr. Dvorak spent several weeks working at the Institute of Biophysics in Rio de Janeiro, Brazil. Dr. Ottesen continued his collaborative work on immunology of filariasis with several groups in Madras, India and Dr. Diamond followed his one year of TDY in Mexico by a three month stay in Japan. Drs. Rosen and Tesh of Pacific Research Section continued their pursuit of arboviruses in the Far East.

The following changes in professional personnel occurred during the past year: Several individuals, such as Drs. Gottlieb and Martin, who had been with us for some years as Staff Fellow and Visiting Associate, respectively, left for academic positions and further training at other institutions. Dr. Kassim who had been a Visiting Fellow started a new job in Nigeria and Dr. MacAuliffe who had been a Staff Fellow decided to take time off with family responsibilities. Newcomers to the LPD included Staff Fellows Drs. Rosenberg, and Rener. We also had new Visiting Fellows from Britain and Israel, Drs. Crane and Epstein and a new Research Associate, Dr. Weil. Dr. Ebrahimzadeh, Prof. of Microbiology from Ahwaz, Iran spent several months with LPD as a Guest Worker and Dr. Mercado returned from a two year assignment from University of California at San Diego.

Due to constraints of space the work on the biology and genetics of schistosome vector snails will move physically to a nearby location in Rockville. Dr. Richards will be joined in his research efforts by Dr. Sullivan who has a special WHO fellowship.

We will miss Dr. Sheffield, who headed the section on Cell Biology and Immunology, but wish him well on his new administrative position with the Extramural Program of NIAID.

A combination of scientific, financial and administrative considerations resulted in the discontinuation of the Pacific Research Section (PRS) in Honolulu as an integral part of the Intramural program of NIAID. This action was certainly not due to any shortcomings in research productivity of PRS; in fact, the Pacific Research Laboratory had an enviable record of findings in parasitic disease and arbovirus epidemiology. Their contributions to the study and understanding of dengue infection

have been highly original and important. The most recent work on vertical transmission in the arthropod vector of several different arboviruses for which this mechanism was not seriously considered will prove to be key factor in their epidemiology and might well open up new ideas in the evolution of these viruses. Some of the PRS work under Dr. Rosen and Tesh will continue to be supported by an NIAID contract, and we wish our former colleagues well in their new administrative relationship. Dr. Wallace will come to Bethesda to join the Scientific Director's Staff and Dr. Dean will take a position elsewhere.

Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - October 1, 1977 through September 30, 1978

RESEARCH ACCOMPLISHMENTS

CELL BIOLOGY AND PHYSIOLOGY OF PROTOZOAN PARASITES

Leishmania: Using macrophages from the bone marrow of hamsters infected with L. donovanii an estimate of 50 hours was made of the mean generation time of these intracellular parasites with rupture of the host cell after 8 - 9 division cycles. Although electron microscopic examination shows that virtually all intracellular amastigotes of this parasite reside and multiply within host cell phagolysosomes, accumulating evidence suggests a dynamic interchange and recycling between the lysosomal-parasitophorous vacuolar system and the plasma membrane of the infected macrophage so that parasite antigens are demonstrable on the infected cell membrane. (Dwyer). Experimental techniques have been developed to study the surface membrane constituents of promastigotes of L. donovanii and isolation and characterization of these membranes. The techniques included labelling with 125_I in conjunction with treatment of cells with various proteolytic enzymes to remove bound 125_I activity as well as other enzyme labelling techniques. In addition, improved methods were devised for isolation and purification of subpellicular membrane microtubules. Sharing of surface membrane antigens by L. donovanii promastigotes and amastigotes has been demonstrated by this technique. These methods should facilitate isolation, immunochemical characterization of specific leishmanial surface membrane antigens and the role of these structures during the course of in vivo or in vitro infection. (Dwyer).

The various aspects of the hexose transport system of L. tropica have been studied and suggest that transport and phosphyorylation activity are closely linked and may be analogous to the bacterial phosphotransferase system. In leishmania a separate transport mechanism for glycerol has been shown. (Gottlieb).

Trypanosomes: One of the most interesting developments in this area has been the production of a vertebrate cell-parasite cell hybrid. Using modifications of cell hybridizing techniques, epimastigotes of T. cruzi were fused with cells of a continuous human cell line. Some lines of the hybrid appear to be stable and show morphologic evidence of fusion and the progeny appears to express T. cruzi antigen. (Crane and Dvorak). Various aspects of parasite-host cell interaction of T. cruzi which have been studied previously were explored with parasites of varying origin. A shorter lag period before onset of multiplication and shorter doubling time were found for the mouse passaged Y strain. Virulence for mice after passage through the insect vector was shown for a strain of T. cruzi that had been grown for about 14 years in cell cultures. (Dvorak). A paradoxical result was obtained in an effort to test ability of T. cruzi to invade cells in the presence of serum from patients with Chagas' disease. Serum from chronic patients who had antibody against T. cruzi actually promoted penetration of host cells. This

enhancement could not be related to presence of either Fc or C3 receptors on host cells but the penetration promoting activity of this serum was significantly reduced by pre-absorption with host cells. (Dvorak and Schmunis).

A technique for formation of plaques in a mammalian cell monolayer infected with the progeny of a single trypanosome has been developed for assessing the ability of different strains of T. cruzi to grow within their host cells. The ability to produce plaques showed a direct linear relationship to inoculum size as estimated by terminal dilution. This technique should be useful in assessing the intrinsic biologic characteristics of different strains of T. cruzi or the organisms resulting from mutation or adaptive manipulations. (Neva).

Amebae: Extensive experience has now confirmed the suitability of the new iron containing medium for axenic growth of E. histolytica which does not require the unpredictable liver extract of the older medium. Growth can now be obtained with a wide variety of strains, with smaller inocula, uniform growth and reduced generation time. Although yeast extract is still required, the medium now is more suitable for further determination for specific growth factors. (Diamond). In collaborative studies with Mexican investigators the influence of serum and iron tissue stores was evaluated in patients with amebic liver abcess. Reduced serum iron levels and serum iron saturation were found in liver abcess patients but similar findings would be expected in patients suffering from bacterial infections as well. Additional controls and patients with intestinal amebiasis are still being studied by Mexican collaborators to better evaluate the findings in patients with extra-intestinal amebiasis. Newborn guinea pigs inoculated directly into the cecum by laparotomy were found to be susceptible to intestinal lesionas by E. histolytica. This finding parallels that of last years work on new born hamsters and mice and may be useful for studying the influence of bacteria for establishment of intestinal lesions in view of previous work with this model. (Diamond, and Mexican collaborators). Virtually all of the respiratory flavoprotein of axenically grown E. histolytica was found to be in a soluble fraction of disrupted organisms. The amount and activity of riboflavin kinase indicates that the parasite probably has the ability to synthesize its own flavonucleotides. (Takeuchi and Claggett). Studies initiated on the uptake of sugars and amino acids by E. histolytica indicated that cysteine which is essential for both growth and maintenance of this organism is rapidly taken up. (Gottlieb).

The agar suspension method for clonal growth of E. histolytica was used to test the sensitivity of this organism to various temperatures and to oxygen. The proposal by others that pathogenic strains of E. histolytica were Con A agglutinable, in contrast to nonpathogenic strains, was confirmed with strains of E. histolytica from this Laboratory. This finding reinforces the concept that pathogenesis is determined by surface membrane properties. (Gillin).

Giardia: Studies have been initiated on Giardia lamblia with the human strain originally isolated by Meyer and provided by CDC workers. The organism grows well axenically in the media developed for growth of E. histolytica. (Diamond). An absolute and specific requirement for L-cysteine

was found with this organism. Surface membrane properties of both cysts and trophozoites of G. lamblia have not been revealing as yet on the basis of lectin binding experiments. (Gillin).

Sarcocystis: Ultrastructural studies of the developmental cycle of Sarcocystis cruzi, which can probably be considered similar to Isospora hominis of man, were undertaken in the dog. This organism was found to invade mucous cells of the intestine where gametogenesis proceeded and the layers which make up the oocyst wall were found to be products of both parasite and host cell. Fertilization occurred by fusion of gamete membrane and transfer of the male chromatin directly into the cytoplasm of the female gamete. (Sheffield).

MALARIA RESEARCH PROGRAM OF THE LPD, NIAID

Free ranging or basic research is oriented for the development of new approaches to the practical problem of global resurgence of malaria. Today, two areas of malaria research seem to offer promise of realistic goals: a) some type of immunization against malaria and, b) biological control of the mosquito vector. Two general approaches have been taken toward the goal of possible immunization against malaria: 1) to gain insight into the mechanism of immunity and immunosuppression in the hope of learning more about immune responses which might either assist or interfere with host defenses,and 2) To isolate and characterize the antigens of sexual and asexual stages of the malaria parasite. With respect to the goal of vector control, physiology and behavior of the Anopheline mosquito are being studied for clues to possible new approaches.

Gametes as immunogens: Highly purified extracellular macrogametes of P. gallinaceum could be prepared using lectin agglutination of host cells. But, as with purified microgametes, such preparations were found to be poorly immunogenetic for transmission blocking immunity in chickens. This was in contrast to the highly effective transmission blocking immunity achieved with preparations of gametes with mixed sexes. (Carter and McAuliffe). Rhesus monkeys immunized with a single inoculation of 10^6 or 10^7 macrogametes in Freund's complete adjuvant have shown persistent transmission blocking immunity for over 2 years. (Gwadz and Green). Studies were taken to the field to look for evidence of transmission blocking antibodies among native Africans in an endemic area for malaria. Such antibodies were not found. In the same study it was shown that surface antigens of macrogametes and merozoites did not share specificities that could be demonstrated by fluorescent antibodies. (Carter, Gwadz and Green). Antisera from immunized Rhesus monkeys are in preparation to compare properties of sexual and asexual stages of the malaria parasite. (Carter, Gwadz, Green and Miller). The continuous in vitro technique of Trager and Jensen is being utilized to try to produce gametocytes in vitro cultures. The production of functional gametocytes has been demonstrated but there is difficulty in doing it reproducibly and at will. Various factors influencing gametocyte maturation are under study. (Carter and Beach).

Factors influencing malaria parasite invasion: Malaria parasites invade red cells (RBCs) by a sequence of steps that include recognition and attachment, junction formation, invagination of the red cell membrane

around the parasite and sealing of the vacuole. In order to show the structural and functional differences of the normal invasion sequence by interaction with Duffy negative RBCs, a method was developed for the isolation of the attachment phase of invasion by cytochalasin treatment of merozoites. (Miller, Aikawa, Johnson and Shiroishi). Cytochalasin-treated merozoites attach only to red cells from a susceptible host although the rate of attachment is the same for Duffy positive and negative RBCs. But junction formation only occurs with Duffy positive RBCs. Therefore, the Duffy associated antigen is required for this step in the invasion sequence. Red cell pyridoxal kinase is higher in Nigerian children with severe malaria than the general population. Malaria parasites lack this enzyme and must depend on the red cell enzyme for conversion of pyridoxal to pyridoxal sulfate. (Martin, Clark and Miller). The enzyme is lacking in red cells of hosts not susceptible to malaria, such as cats, dogs, horses and cows.

Physiology of feeding and oviposition in anophelines: In addition to its role in egg development, the steroid hormone, ecdysone, also acts on the mosquito nervous system causing inhibition of oviposition and biting behavior. These effects can be induced at any time by administration of commercially prepared ecdysone. Other hormones such as matrone also elicit changes in behavior as well as the physiological changes with which they have been traditionally associated. (Beach).

Immune response in malaria: The role of the spleen in host defense against malaria has stimulated investigations of the importance of this organ in intravascular clearance of parasitized erythrocytes in immune animals in efforts of identifying cell populations responsible for host defense and immunosuppression. Intravascular clearance of parasites seems to depend upon interactions of infected red cells with the spleen on the basis of rheologic rather than immunologic factors. (Quinn and Wyler). Since reconstitution with spleen cell suspensions permits splenectomized animals to survive their malaria infections whereas non-reconstituted mice die, this *in vivo* model appears to be useful for studying the role of spleen cell populations in host defense. (Oster and Wyler).

HELMINTHIC INFECTIONS

Experimental studies on pathogenesis: Hepatic fibrosis in rabbits infected with *Schistosoma japonicum* was found to bear similarities to Symmers' fibrosis of individuals with severe Manson's schistosomiasis. In early stages the portal fibrosis was associated with minimal distortion of liver architecture but in later stages the fibrosis regressed and marked distortion of lobular architecture occurred. This latter feature is recognized but not frequently seen in the human disease. Hepatic function was well preserved as in the human disease. (Cheever). Isolated schistosoma egg granulomas were found to secrete substances *in vitro* which were capable of stimulating fibroblast proliferation and collagen synthesis of mammalian cells. This finding may provide a link between the delayed hypersentivity reaction around eggs and subsequent fibrosis of the liver. (Wyler, Wahl, Wahl and Cheever).

Schistosomal antigens: Studies of serotonin in schistosomes, presumably an important neuro-transmitter substance for this parasite, have shown uptake to occur by means of a membrane receptor on the schistosomal

surface. (Catto and Ottesen). In vitro culture of adult schistosomes exposed to various precursor substances has been used as a technique for studying secretory products of the worms. The number of substances secreted that are labelled with N-acetylglucosamine are relatively large compared to the one or two substances found in glucose labeled secretions. (Nash). A carbohydrate antigen has been isolated from S. mansoni eggs and characterized. This antigen differs from previously described schistosome egg antigens, and antibodies to this carbohydrate egg antigen were found to be present in experimental S. mansoni infections. (Boctor and Nash). When antibody responses to cercarial vs adult worm schistosome antigens were compared by means of the ELISA test it was found that patients with chronic schistosomiasis had higher antibody levels to adult worm antigens and patients with acute schistosomiasis had higher titers to cercarial antigens. (Lunde, Ottesen and Cheever).

Host response to schistosome infections: The mechanism(s) of suppression of cellular response to specific parasite antigens previously found in chronic schistosome infections was found related to serum inhibitory factors and monomuclear suppressor cells removable by glass wool adherence. High levels of immune complexes were demonstrated in essentially all patients with acute schistosomiasis but much less commonly in patients with chronic infections. As acute cases were followed sequentially for more than a year the levels of immune complexes either fell spontaneously or after treatment. (Ottesen and Lawley).

Immune respones to filarial infections: Studies in India showed that while all patients with filariasis have high IgE levels and anti-filarial IgE antibodies, patients with tropical pulmonary eosinophilia were most highly sensitized to all filarial antigens but particularly those derived from microfilaria. Furthermore, there was marked species specificity to these antigens which supports the contention that human rather than animal filarial parasites cause tropical pulmonary eosinophilia. (Ottesen and Indian collaborators).

Vector snail genetics: Nine different pigment phenotypes have been identified which can serve as genetic markers in the schistosome vector snail Biomphalaria glabrata. An additional variation in susceptibility patterns to S. mansoni has been found in B. glabrata snails. Some that are susceptible as juveniles and become resistant during periods of maximum egg production may revert again to susceptibility in old age. The infectivity of different strains of S. mansoni from Puerto Rico for a standard snail host indicate five or more genetic variations of this parasite. (Richards).

NON-PARASITIC BIOCHEMISTRY

Bioenergetics of bovine adrenal gland chromaffin granules: In contrast to previous findings that uncouplers of oxidative phosphorylation were potent inhibitors of the release reaction in granules, anion channel blockers which also block the release reaction, were ineffective as uncouplers. During these studies it was found that catalase, but not peroxidase, was associated with the granule fraction. (Weinbach and Pazoles of NIAMD).

PACIFIC RESEARCH SECTION IN HAWAII

Transovarial transmission of mosquito-borne viruses: Transovarial transmission by mosquitoes of Japanese encephalitis, dengue, yellow fever, and several other flaviviruses was demonstrated in the laboratory. These findings, after more than 75 years of negative or unconfirmed positive reports, probably will lead to re-examination of the epidemiology of all mosquito-borne viruses. If it is shown that transovarial transmission is an important factor for virus maintenance in nature, new strategies for disease control or eradication may be possible. (Rosen and Tesh).

Bedbugs and hepatitis B virus: With the recent interest in bedbugs as biologic vectors of arboviruses and data suggesting that they may be more than just mechanical vectors of hepatitis B virus, the experimental work described in this project, whether or not positive results are obtained, should prove of considerable interest. (Tesh).

Swine influenza: Epidemiologic data from Hawaii continues to support the hypothesis that swine influenza is maintained in nature by chronic or latent infection in swine - not involving lungworms. Experimental attempts to clarify the mechanism by which this occurs have so far proven unsuccessful. (Wallace).

Kawasaki disease: The largest epidemic of Kawasaki disease ever reported in the United States has recently occurred in Hawaii. Epidemiologic and laboratory studies of the outbreak may provide clues as to the etiology of the disease. (Dean).

Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - October 1, 1977 through September 30, 1978

HONORS AND AWARDS

Dr. Louis H. Miller serves on the editorial board of the following journals: Experimental Parasitology, Journal of Parasitology, and Journal of Molecular Medicine. Served as a consultant to the following organizations and for the following purposes: a) The Institute of Medicine in their study on International Health Activities; b) Agency for International Development for their malaria research programs; c) Rockefeller Foundation Babesia Vaccine Program; d) Indian Council for Medical Research for review for possibilities of collaborative research.

Dr. Dvorak served as a member of the WHO Scientific Working Group on Chagas' Disease at a meeting in Buenos Aires, Argentina, 14-18 November 1977. Dr. Dvorak was a Visiting Professor at the Federal University of Rio de Janeiro, in Brazil from 8 October to 23 November 1977. During this time he gave a number of lectures.

Dr. R. W. Gwadz awarded Public Health Service Commendation Medal in 1978 for work on experimental malaria vaccines. He served as a member of the Site Visit Committee for NIAID to visit ICMR units in Pakistan and Malaysia.

Dr. David Wyler served as a member of the NIAID Clinical Research subpanel. He was invited to speak at a WHO workshop on the role of the spleen and parasitic diseases in Geneva, July 12-14, 1978 and was elected to membership in the American Federation for Clinical Research.

Dr. Dennis Dwyer was re-appointmented as adjunct Associate Professor of Parasitology at Rockefeller University, New York City and Associate Professor of Zoology at the University of Massachusetts (Amherst). He was appointed to the Editorial board of The Journal of Protozoology.

Dr. Harley Sheffield was elected President of Helminthological Society of Washington for 1977-1978.

Dr. Franklin A. Neva serves on the editorial boards of American Journal of Tropical Medicine and Hygiene and the Journal of Infectious Diseases.

Dr. Eric Ottesen was an invited participant for a Brook Lodge Conference on Escape from Immune Surveillence: The Interface between Immune Mechanisms and Disease.

Dr. Ted Nash was an invited participant for a WHO meeting of the Steering Committee on Schistosomiasis - Subjection on Immunodiagnosis.

Dr. Leon Rosen was invited to present papers on transovarial transmission of Japanese encephalitis virus by mosquitoes at the Third International Congress of Virology (The Hague, Netherlands), at a meeting of the virus panel of the U. S. - Japan Cooperative Medical Science Program (Osaka, Japan), and at a symposium on transovarial transmission of viruses by mosquitoes at the Annual Meeting of the American Society of Tropical Medicine and Hygiene (Chicago).

Dr. Robert Tesh was invited to present a paper on transovarial transmission of Japanese encephalitis and yellow fever viruses by mosquitoes at a working conference on "Dengue Fever in the Caribbean" (Montego Bay, Jamaica), sponsored by the World Health Organization.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00091-16 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Genetics, Biology, and Control of Snail Intermediate Hosts of Schistosomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C.S. Richards, Parasitologist, LPD, NIAID

Other: O. Kassim, Visiting Fellow, LPD, NIAID

COOPERATING UNITS (if any) Dr. K.J. Lie and Dr. Donald Heyneman, The George Williams Hooper Foundation, University of CA. Dr. Thomas C. Cheng, Institute for Pathobiology, Lehigh University. Dr. Fredrico S. Barbosa, Faculdade de Ciencias da Saude, Universidade de Brasilia. Dr. Ernesto Ruiz, San Juan Laboratories,

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host-Parasite Relations Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
36/12	18/12	18/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Genetic variations occur in susceptibility to infection in Biomphalaria glabrata and infectivity in Schistosoma mansoni. Studies indicate at least 12 genetically different susceptibility types in B. glabrata. Three S. mansoni isolations from chronic Puerto Rican cases believed to have been reinfected, one isolation from St. Lucia, and one from Egypt have each yielded by selection two genetically different substrains of S. mansoni.

A gene regulating mantle pigment in B. glabrata has three alleles; mantle spotting, coalesced mantle pigment, and absence of mantle pigment. Absence of pigment is recessive, and snails heterozygous for spotting and coalescence show a combined expression of both alleles, so this gene involves four phenotypes. Since the gene for albino, blackeye, or wildtype pigmentation is located on a different chromosome, we can now distinguish nine different pigment phenotypes serving as genetic markers in B. glabrata.

In some genetic types of B. glabrata, snails are susceptible as juveniles,

PHS-6040
(Rev. 10-76)

Cooperating Units (Continued)

Bureau of Laboratories, CDC, San Juan, P.R. Dr. David Woodruff, Dr. Phil LoVerde, Ms. Madeleine Fletcher, and Mr. Dennis Minchella, Dept. of Biology, Purdue University, Mr. Walter N. Stewart and Dr. Ned Feder (LEP, NIAMD). Dr. Roger M. Cole and Mr. Terry Popkin (LSD, NIAID). Dr. Christopher Bayne, Dept. of Biology, Oregon State University.

Summary (Continued)

resistant during the period of maximum egg production, and revert to susceptibility in old age. This is pertinent to snail and parasite survival in an endemic area, to studies on the mechanisms of resistance, and to experimental research and field surveys.

Project Description:

The objective is to study molluscan and trematode genetics with particular attention to applications to control measures. To study prospective agents for biological control of intermediate hosts of schistosomes. To study the biology of the snails with the aim of improving the efficiency of control measures. To study the contributions and complications of molluscan diseases and molluscan genetics to bio-medical research. To study the causes of tumors in mollusks. To study host-parasite interaction and molluscan cellular defense mechanisms. To study snail amebocytes, particularly as related to defense against parasites.

Individual snails are exposed to various strains of Schistosoma mansoni, and selection and crossing experiments conducted to investigate further the genetic aspects of molluscan susceptibility to infection. Results of penetration of individual miracidia of S. mansoni in different snail types are observed and selection and crossing experiments conducted to investigate the genetic aspects of schistosome infectivity.

Establishing genetic stocks of B. glabrata,particularly different types of susceptibility to S. mansoni, provides material for bio-chemical, biological and cytogenetic intraspecific analyses. Such stocks have been provided on request to many other investigators.

Need for evaluation of the role of genetics in snail control continues. Resistant B. glabrata may prove useful in biological control of schistosomiasis by dilution or replacement of populations of susceptible snails in endemic areas. Resistant B. straminea may be effective in interspecific competition. Samples of genetically resistant snails of these two species have been sent to Dr. Barbosa, University of Brasilia, for collaborative laboratory and field studies along these lines. Genetic resistant and susceptible stocks of B. glabrata have been provided Mr. Dennis Minchella, Purdue University, for laboratory competition studies. Progress in understanding causes of susceptibility or resistance of B. glabrata to infection with S. mansoni accelerates the prospect of safe field testing of resistant snail stocks for control.

Project Description (Continued)

Formal genetic studies on B. glabrata yielded further information on the mutant causing coalescence of black mantle pigment in wildtype and blackeye snails. Studies in cooperation with Mr. Walter N. Stewart and Dr. Ned Feder (LEP, NIAMD) have shown that coalescence, spotting and absence of either are determined by three alleles of the same gene. Coalescence and spotting are both dominant over absence of such pigment. Snails heterozygous for the coalescence and spotting alleles show a combined expression of both alleles, thus this gene involves four phenotypes. Since the gene determining wildtype, blackeye, or albino pigmentation is located on a different chromosome, we now can distinguish nine different pigment phenotypes in B. glabrata. Mantle spotting (dominant) and absence of spotting have also been demonstrated in Biomphalaria tenagophila from Brasil and Biomphalaria alexandrina from Egypt, providing genetic markers for studies on these intermediate hosts for S. mansoni.

Amebocytes play an important role in the defense of B. glabrata against infection by S. mansoni. Studies on the occurrence of genetic proliferative amebocytic accumulations are continuing. Dr. Christopher Bayne of Oregon State University is studying, in culture media, cells from hemolymph of some of the B. glabrata with amebocytic accumulations and their relation to S. mansoni sporocysts.

Collaborative studies on interaction between B. glabrata and S. mansoni are continuing, with Dr. Lie and Dr. Heyneman of the Hooper Foundation and Dr. Cheng of Lehigh University, including hemolymph enzyme analyses and effects of exposures to echinostome larvae on resistance of snails to S. mansoni. These studies showed the strain specificity of the relation of a B. glabrata stock to compatible or incompatible miracidia, thus supporting the results of studies by Dr. Kassim in our laboratory using autoradiography and mixed compatible and incompatible miracidia.

In collaborative studies at Purdue University Dr. David Woodruff and associates are pursuing allozyme studies on our stocks of B. glabrata differing genetically in susceptibility, with preliminary results showing differences in allozyme patterns.

Studies on a variety of tumors observed in B. glabrata, many of which are genetic, will be continued.

Microsporida, amebae, and bacteria cause extensive damage in some snail hosts. These and other snail diseases need to be examined for potential human infection, and prospective use as agents in biological control of snails. It is essential to be aware of the occurrence of these infections and their relations with the snails and other parasites to plan, conduct, and interpret experimental research on snails with validity. In collaboration with Dr. Roger M. Cole and Mr. Terry Popkin (LSD, NIAID) attempts are being made to culture bacteria infecting Bulinus jousseaumei.

Project Description (Continued)

Genetic variations in infectivity for B. glabrata in strains of S. mansoni of Puerto Rican origin are being studied in collaboration with Dr. Ernesto Ruiz (San Juan Laboratories, CDC, P.R.). B. glabrata of several genetically characterized susceptibility types are being exposed to each S. mansoni strain. Strains isolated from three chronic cases believed to have been reinfected have each yielded two genetically different substrains with respect to infectivity for snails. Studies to date suggest the occurrence in Puerto Rico of five or more genetic variations of S. mansoni. Studies with some of these strains indicate differences in the course and intensity of infections in mice.

Tests on adult and juvenile B. glabrata indicate the occurrence of at least 12 genetically different susceptibility types and suggest that susceptibility in juvenile and old snails with resistance to S. mansoni in snails during the period of egglaying may not be uncommon. This genetic susceptibility pattern is pertinent to survival of both snail and parasite populations, to studies on the mechanism of resistance, and to methods employed in both experimental research and field surveys.

Genetic studies on Biomphalaria species will be continued to: a) seek additional genetic markers; b) expand our knowledge of linkage groups: and c) test additional stocks of B. glabrata and strains of S. mansoni as available. Genetic studies on S. mansoni will be continued to establish different genetic infectivity strains and elucidate the genetics of infectivity.

Publications:

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Cole, R.M., Richards, C.S., and Popkin, T.J.: A novel bacterium infecting an African snail. Jour. Bact., 132: 950-966, 1977.

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Publications (Continued)

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00092-12 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

The pathogenesis of schistosome infections in mammalian hosts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.W. Cheever	Asst. Chief, Laboratory of Parasitic Diseases Head, Section on Host-Parasite Relations	NIAID
Other:	T.E. Nash	Medical Officer	LPD NIAID
	E.A. Ottesen	Medical Officer	LPD NIAID

COOPERATING UNITS (if any) Foundation for Research and Education; Department of Pathology, Hospital Prof. Edgard Santos, Salvador, Brazil; Department of Pathology, Cairo University Faculty of Medicine, Cairo, Egypt; Department of Zoology, University of Georgia; Laboratory of Statistical and Mathematical

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host-Parasite Relations

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
30/12	12/12	18/12

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Schistosome infections are studied quantitatively in man and in experimental animals. Experimental Schistosoma japonicum infections in rabbits produce severe hepatic fibrosis which shares some anatomic similarities with Symmers' clay pipestem fibrosis in man. Collagen synthesis and degredation in these livers are being examined in collaborative studies. Intestinal, but not hepatic, lesions have varied greatly with the strain of schistosome used to infect rabbits. In infected Egyptian patients examined at autopsy, cor pulmonale attributable to schistosome infection was always accompanied by schistosomal pulmonary arteritis and was seen only in cases with S. mansoni infection and Symmers' fibrosis.

PHS-6040
(Rev. 10-76)

Cooperating Units, continued:

Methodology, DCRT; Division of Clinical Oncology, University of Wisconsin Medical School. Department of Gastroenterology, WRAIR.

Project Description:

The nature and pathogenesis of the lesions produced in mammalian hosts by schistosomes of medical importance are studied in man and experimental animals. Schistosome infections are quantitated by measurement of egg passage in the excreta and egg accumulation in host tissues.

Symmers' fibrosis of the liver is responsible for most of the morbidity and mortality caused by S. mansoni and S. japonicum infections. The chimpanzee has been the only adequate model for this hepatic lesion. Rabbits with heavy S. japonicum infections develop lesions grossly and microscopically similar to Symmers' fibrosis in man. In contrast to patients with Symmers' fibrosis, the rabbits show extensive hyperplasia of bile ductules and marked septal fibrosis producing a histologic picture resembling postnecrotic cirrhosis with marked portal fibrosis in many animals. Hepatic function is well maintained in these animals. The model seems an imperfect one, at least from the anatomic findings. Synthesis and degredation of collagen in the livers of these rabbits is being examined in collaboration with Drs. Goldner and Dunn at WRAIR.

Examination of antibody dependent cell mediated cytotoxicity of sera from infected persons against S. mansoni schistosomules gave inconsistent results from week to week and this work has been temporarily halted.

During the next year the descriptive pathology, parasitology and pathology of the rabbit model should be completed. Use of the model for examination of collagen metabolism will be expanded. Examination of the relation of egg antigens to the production of fibroblast stimulating factor by isolated granulomas and lymphoid cells will continue with Wyler et al (Z01 AI 00109-05). A visit to the Philippine Islands is planned to examine the possibilities of conducting a quantitative post mortem study of S. japonicum infection in man.

Understanding of the lesions cause by schistosomiasis in man, the associated morbidity and their relation to intensity of infection are basic to planning control of the disease and to treatment of patients. If light infections are unimportant, then these should not be treated (all available dugs are toxic) and control measures aimed at reducing infection intensity should be vigorously supported.

Publications:

Kamel, I.A., Cheever, A.W., Elwi, A., Mosimann, J.E. and Danner, R.: Schistosoma mansoni and S. haematobium infections in Egypt. I. Evaluation of techniques for recovery of worms and eggs at necropsy. Am. J. Trop. Med. Hyg. 26: 696-701, 1977.

Cheever, A.W., Kamel, I.A., Elwi, A.M., Mosimann, J.E. and Danner, R.: Schistosoma mansoni and S. haematobium infections in Egypt. II. Quantitative parasitological findings at necropsy. Am. J. Trop. Med. Hyg. 26: 702-716, 1977.

Cheever, A.W., Kamel, I.A., Elwi, A.M., Mosimann, J.E., Danner, R. and Slippel, J.E.: Schistosoma mansoni and S. haematobium infections in Egypt. III. Extrahepatic pathology. Am. J. Trop. Med. Hyg. 27: 55-75, 1978.

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Mosimann, J.E., Malley, J.D., Cheever, A.W. and Clark, C.B.: Size and shape analysis of schistosome egg-counts in Egyptian autopsy data. Biometrics (In press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00093-03 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of the Immunologic Response to Helminth Infections

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: E. A. Ottesen — Senior Investigator, LPD, NIAID

Other:
R. D'A. Gusmao — Visiting Fellow, LPD, NIAID
B. Catto — Research Associate, LPD, NIAID
F. A. Neva — Chief, Laboratory of Parasitic Diseases, NIAID
T. Lawley — Medical Investigator, NCI

COOPERATING UNITS (if any) San Juan Laboratory, Center for Disease Control (Dr. R. Hiatt); Tuberculosis Chemotherapy Center, Madras, India (Dr. S.P. Tripathy); Medical College of Madras, India (Prof. K.V. Thiruvengadam).

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host-Parasite Relations

INSTITUTE AND LOCATION
NIAID, Bethesda, MD.

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50/12	26/12	24/12

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major goals of this project are to characterize the host's immune response to helminth infections and to relate the findings to the pathogenesis of clinical disease. Chronic filariasis and schistosomiasis are both characterized by cellular immune hyporesponsiveness to parasite antigens which may play a role in the persistence of the parasite within the host. The mechanisms involved in this hyporesponsiveness include both serum inhibitory factors and mononuclear suppressor cell populations. Profound immunologic hyperresponsiveness of the immediate type (IgE) characterizes the asthma-like tropical eosinophilia syndrome of human filariasis. Here the most important antigens (as determined by basophil histamine release experiments) appear to be those derived from microfilaria. In acute schistosomiasis not only is there marked cellular and humoral immune activity but also the presence of large numbers of circulating immune complexes which may be important in the syndrome's pathogenesis.

Larval schistosomes (schistosomules) have been demonstrated to contain the biogenic amine, serotonin. Serotonin uptake by these organisms appears to be via a membrane receptor.

PHS-6040
(Rev. 10-76)

Project Description:

The character and evolution of the immune response in man and experimental animals has been studied during and following infection with parasitic helminths.

Filariasis. The questions of why some individuals in an endemic area become infected with filaria (bancrofti) while others appear to resist it and of why even among infected individuals the clinical manifestations of disease can be so varied have led to a search for the relevant immunologic parameters both in field studies of human filariasis and in work on a laboratory model of the disease. Field studies in the Cook Islands (Project #JO233-I-74) and in India (Project #76-I-231) have shown that in endemic areas the greatest cellular immune reactivity (lymphocyte transformation) and the highest production of antibodies to filarial antigens (determined with ELISA techniques) occur not in infected patients but in those equally exposed to infective parasites but who have not acquired the infection. In fact, there exists in infected individuals a state of specific cellular immune unresponsiveness to filarial antigens which appears to be determined both by serum inhibitory factors and by suppressor cells which can be identified among the adherent subpopulation of mononuclear peripheral blood cells. The nature of these suppressive elements is currently being explored. (Ottesen)

Tropical eosinophilia (TE) is a form of filariasis characterized by chronic lung disease with episodes of paroxysmal nocturnal asthma, profound blood eosinophilia, markedly elevated IgE levels and high filarial antibody titers. Using the in vitro correlate of IgE-mediated allergic responses (ie. the histamine release reaction of IgE-coated basophils challenged in vitro with the sensitizing allergen) and a radioenzymatic assay for the detection of histamine, we have documented and quantified the high degree of allergic sensitization to filaria in patients with TE (compared to those with other forms of filaria diseases). The especial hypersensitization to microfilariae and their products may be most important in determining the symptoms and pathology associated with this syndrome. At the other extreme, individuals with circulating microfilaria who might be expected also to be allergically sensitized were parodoxically hyporesponsive antigens derived from microfilariae, and the mechanisms underlying this hyporesponsiveness are currently under study (Ottesen, Neva).

Previous observations with an experimented model of Brugia pahangi filariasis in Lewis rats indicated that the early development of IgE anti-filarial antibodies was associated with protection from infection. Studies to define the specificity of these antibodies and their effect in passive transfer experiments are currently in progress. (Gusmao, Ottesen).

Schistosomiasis. Previous studies in our laboratory have defined the progressive loss of parasite antigen-specific cellular immune responsiveness (lymphocyte transformation) in patients whose schistosome infections evolve

from the acute to chronic stages. As we have explored the mechanisms involved this modulation of antigen responsiveness, it has become clear that just as in the patients with filariaisis, individuals with chronic S. mansoni infections have inhibitory elements both in their serum and in their adherent mononuclear cell populations. The serum and cell suppressive effects may be additive and their natures are being investigated (Ottesen).

Serum samples from patients followed closely for almost two years after developing acute schistosomiasis have been studied for the presence of Cl_q binding immune complexes. All patients with symptomatic acute schistosomiasis had circulating immune complexes which either declined gradually as the infection became chronic or declined rapidly after treatment. The finding of circulating complexes in patients with chronic infection was much less common (2 of 11 patients) than it was in acutely infected individuals. Studies to determine the potential role of these complexes in the pathogenesis of the acute syndrome are in progress (Lawley, Ottesen).

Biochemical studies on the major neurotransmitter of schistosomes (ie. serotonin) have defined the presence of this amine in the schistosomule stage of the organism. Furthermore, the kinetics of serotonin uptake have been studied using a double label, radioenzymatic technique and current evidence suggests that this uptake occurs through a serotonin receptor on the surface of the schistosomule (Catto, Ottesen).

Eosinophils in the host response to helminths. It has been noted by others that eosinphilia often follows treatment of helminth infections. We confirmed these observations in patients with either schistosomiasis mansoni or bancroftian filariasis. Then by studying the post treatment eosinphilic responses in 47 individuals with bancroftian filariasis we found that the magnitude of this post-treatment eosinophilia depended directly on the number of microfilaria in the circulation immediately prior to initiation of therapy with diethylcarbamazine. Efforts to detect circulating eosinophilopoeitic factors in these patients during the period of their development of eosinophilia are under way (Ottesen).

Publications:

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Neva, F. A., and Ottesen, E. A.: Tropical (Filarial) Eosinophilia. New Eng. J. Med.: 298: 1129-1131, 1978.

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Lunde, M. N., Ottesen, E. A., and Cheever, A. W.: Serological Differences Between Acute and Chronic Schistosomiasis mansoni Detected by Enzyme Linked Immunosorbant Assay (ELISA). Am. J. Trop. Med. Hyg. (in press).

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Weller, P.F.: Cell-Mediated Immunity in Experimental Filariasis: Lymphocyte Reactivity to Filarial Stage-Specific Antigens and to B- and T-Cell Mitogens during Acute and Chronic Infection. Cell. Immunol. 37: 369-382, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00094-19 LPD

PERIOD COVERED
September 30, 1977 to October 1, 1978.

TITLE OF PROJECT (80 characters or less)

Studies of Entamoeba histolytica and other Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. S. Diamond	Head, Parasite Growth and Differentiation Section, LPD, NIAID
Other:	F. D. Gillin	Senior Staff Fellow, LPD, NIAID
	D. B. Keister	Biologist, LPD, NIAID
	E. C. Weinbach	Head, Physiology and Biochemistry Section, LPD, NIAID
	T. Takeuchi	Visiting Associate, LPD, NIAID
	C. F. T. Mattern	Medical Officer, LVD, NIAID

COOPERATING UNITS (if any) Laboratory of Viral Diseases, NIAID; American Type Culture Collection, Rockville, Md.; Hospital General, Centro Medico Nacional, I.M.S.S., Mexico City.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Parasite Growth and Differentiation

INSTITUTE AND LOCATION
NIAID, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
48/12	15/12	33/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

TYI-S-33 (and a variant BI-S-33) devised for the axenic cultivation of Entamoeba histolytica and other Entamoeba has proved, during 17 months of use, to be clearly superior to TP-S-1 medium in supporting the growth of a variety of Entamoeba species. The results of studies conducted with 33 patients, Hospital General, CMN, I.M.S.S. Mexico City, diagnosed as having hepatic amebic disease suggests that the human host, insofar as the management of iron is concerned, responds to amebic infection as it does to bacterial and fungal infections. Cecal lesions have been produced with axenically cultivated E. histolytica for the first time in an experimental animal (newborn guinea pig). Giardia lamblia can be serially cultivated axenically in either TP-S-1 or TYI-S-33 medium providing these media are sterilized by filtration. No growth is obtained in autoclaved media. On the otherhand, E. histolytica will grow in these media regardless of the method of sterilization. However, growth is at a different rate.

PHS-6040
(Rev. 10-76)

Project Description:

The objectives of this project are 5 fold: 1) to develop and refine techniques for the axenic cultivation of E. histolytica, related Entamoeba and other parasitic protozoa; 2) to determine their nutritional requirements in vitro and to define the physical and chemical conditions for optimal growth; 3) to study the mechanisms of their pathogenicity; 4) to study and characterize the viruses of E. histolytica; 5) to devise new or improve existing methods for the freeze-preservation of these parasites at cryogenic temperatures and to study the mechanisms involved in resistance to freeze-preservation.

TYI-S-33 medium (and a variant BI-S-33) devised for the axenic cultivation of E. histolytica and other Entamoeba has proved to be far superior to TP-S-1 medium which was originally developed in this laboratory and presently is the most widely used culture medium for growing these protozoa. Smaller inocula are required to maintain stock cultures, yields of amebae are substantially greater, growth is more uniform, and especially in the case of E. histolytica the generation time has been reduced by one-half to two-thirds.

Studies to simplify the medium have been initiated. The development of a defined replacement for yeast extract, the most variable of the three biological products used in the medium, has been given first priority. Although this goal has not been attained, our investigations indicate that the following substances will be important constitutents of the replacement: A mixture of nucleic acids, the essential amino acids of MEM tissue culture medium, B vitamins, and a mixture of several Tween compounds, linoleic acid and sodium taurocholate.

The nutritional immunity studies aimed at identifying the role of iron in the pathogenesis of human amebic disease continues. A clinical and laboratory study initiated last year in collaboration with staff members of the Hospital General, Centro Medico Nacional, I.M.S.S., Mexico City, seeks as an initial approach to the problem to answer the question, Does the human host, insofar as the management of iron is concerned, react to invasion of E. histolytica in any way which parallels its response to bacterial and fungal invaders? To this end, the serum iron levels, total iron binding capacity (TIBC) of the serum, and the level of iron saturation of circulating transferrin in patients with amebic liver abscess have been measured. To date, the data gathered from 33 males diagnosed as having hepatic amebic disease on the basis of clinical, radiological, serological, and liver scanning studies revealed that their iron levels were approximately one-half that of healthy controls (n=24), and the TIBC and % saturation of transferrin was one-third less than the controls. Since, these findings are to be expected in patients suffering from bacterial and fungal infections, it would appear on the basis of analogy that humans manage iron in an amebic infection as they do in other infectious diseases. Data from an

additional 33 male and 10 female patients are presently being collated.

Special emphasis has been given, and will continue to be given, to the study of patients with amebic liver abscess because of the seriousness of the disease and the availability of material for study at the Hospital General. However, amebic colitis is not being neglected and patients with this disease are also studied as they become available. In addition, asymptomatic, serologically negative, cyst passers are being studied in an effort to determine whether mere infection of the intestine can initiate the mechanisms whereby the host withholds iron from the infectious agent (Diamond, Pizzuto, Gallegos, Landa).

The production of cecal lesions with axenically cultivated E. histolytica in an experimental animal has been accomplished for the first time. Newborn guinea pigs were employed and the amebae were inoculated directly into the cecum via laparotomy. Since, adult guinea pigs are refractory to infection with axenized amebae, but are susceptible to infection with amebae grown in association with bacteria, the two models (newborn and adult) should be useful for studying the influence of bacteria in establishing intestinal lesions, and age related resistance to intestinal infection. (Diamond, Tanimoto and Martinez-Palomo).

Crude extracts obtained from 12 strains of E. histolytica cultivated axenically and exhibiting different degrees of virulence were evaluated for a variety of cytochrome c reductase activities by allowing the enzymes to act on specific substrates in the presence of cytochrome c. In general, the enzyme activities were similar for virulent and avirulent strains. One strain, HK-9, of intermediate virulence, had significantly higher activities.

Studies on the axenic cultivation of Giardia lamblia have been initiated. A strain isolated by Meyer (University of Oregon) and obtained from Visvesvara (CDC) growing in TP-S-1 medium has been established in this laboratory. TP-S-1 and the recently devised TYI-S-33, both developed for the axenic cultivation of E. histolytica, are being used to maintain the giardia. Interesting differences between the species have been observed in their growth response to these media. Entamoeba histolytica will grow in both media whether sterilized by autoclaving or filtration, but at different rates. Giardia lamblia will grow only in filter-sterilized medium. Moreover, while the growth of E. histolytica in TYI-S-33 is far superior to that obtained with TP-S-1, the reverse is true for G. lamblia. Future studies will attempt to identify the factor(s) responsible for these differences. (See Project No. Z01 AI 00160-02-LPD for results of collaborative studies with F. Gillin on the biology of E. histolytica and G. lamblia).

For results of collaborative studies with investigators in other sections see the following: Project No. Z01 AI 00098-21-LPD, Aerobic energy metabolism of E. histolytica; Project No. Z01 AI 00015-11-LVD, E. histolytica - Studies on virulence, toxin production, and viruses.

Publications:

Takeuchi, T. Weinbach, E. C., and Diamond, L. S.: Entamoeba histolytica: Localization and characterization of phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase, and glycogen synthase. Exp. Parasitol. 43: 115-121, 1977.

Mattern, C. F. T., Keister, D. B., Daniel, W. A., Diamond, L. S., and Kontis, A. D.: Viruses of Entamoeba histolytica VII. Novel beaded virus. J. Virol. 23: 685-691, 1977.

Diamond, L. S., Harlow, D. R., and Cunnick, C. C.: A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Trans. Roy. Soc. Trop. Med. & Hyg. 72. (In press).

Diamond, L. S., Harlow, D. R., Phillips, B. P., and Keister, D. B.: Entamoeba histolytica, iron and nutritional immunity. Arch. Invest. Med. (In press).

Diamond, L. S., Tanimoto-Weki, M., and Martinez-Palomo, A.: Production of cecal lesions in newborn guinea pigs with axenically cultivated Entamoeba histolytica. Arch. Invest. Med. (In press).

Mattern, C. F. T., Keister, D. B., and Diamond, L. S.: Experimental Amebiasis IV: Amebal viruses and the virulence of Entamoeba histolytica. Amer. J. Trop Med. & Hyg. (In press).

Gillin, F. D., and Diamond, L. S.: Clonal growth of Entamoeba histolytica and other Entamoeba in agar. J. Protozool. (In press).

Gillin, F. D., and Diamond, L. S.: Clonal growth of Entamoeba in agar: Some applications of this technique to the study of their cell biology. Arch. Invest. Med. (In press).

Weinbach, E. C., Claggett, C. E., Takeuchi, T., and Diamond, L. S.: Biological oxidations and flavoprotein catalysis in Entamoeba histolytica. Arch. Invest. Med. (In press).

Takeuchi, T., Weinbach, E. C., Gottlieb, M. and Diamond, L. S.: Mechanism of L-serine metabolism in Entamoeba histolytica. Comp. Biochem. Physiol. B. Comp. Biochem. (In press).

Takeuchi, T., Weinbach, E.C., and Diamond, L.S.: Entamoeba histolytica: Localization and characterization of phosphorylase and particulate glycogen. Exp. Parasitol. 43: 107-114, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00097-20 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Pathological Physiology and Histochemistry of Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Teresa I. Mercado Research Physiologist, LPD, NIAID

COOPERATING UNITS (if any)
On sabbatical leave until June 30, 1978, at Department of Biology, University of California, San Diego, Laboratory of Dr. George Fortes.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12/12	12/12	

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The period of sabbatical training at the Department of Biology, University of California, San Diego, was continued until June 30, 1978. Studies on the function of biological membranes were continued employing the plasma membrane of human red blood cells. The activity of sodium, potassium, adenosine triphosphatase (Na^+- K^+- ATPase), the enzymatic expression of the principal cation-transport system of eucaryotic cells, was studied particularly in relation to membrane sidedness. Sealed membrane vesicles, oriented right-side-out (RO) or inside-out (IO), were used in order to assess the enzymic interactions at the two membrane surfaces. The techniques employed in this type of work were particularly useful for future research employing parasite membranes and/or membranes of parasitized tissues.

PHS-6040
(Rev. 10-76)

Project Description:

Outdated blood bank red blood cells were thoroughly washed in isotonic sodium phosphate buffer (PBS) and hemolyzed under controlled conditions of hypotonicty, temperature, and pH. After all the hemoglobin was removed the resulting unsealed ghosts were incubated in very low ionic strength alkaline buffer to induce endocytosis by ion deprivation (reduction in the concentration of electrolytes, divalent cations, and hydrogen ions) or exocytosis, if vesiculation was produced in the presence of magnesium ions. Subsequent gentle homogenization of such preparations resulted in the separation of RO and IO vesicles. Sealed vesicles were then separated from the unsealed by isopycnic density gradient centrifugation employing dextran. To assess the sidedness and sealing of the vesicles their accessibility to certain substrates was examined. For example, acetylcholine, to determine the activity of the externally-located enzyme, acetylcholinesterase, and the reduction of nicotinamide adenine dinucleotide (NAD) to test for the activity of the internally-located enzyme, glyceraldehyde-3-phosphate dehydrogenase. Fairly pure preparations were obtained; RO vesicles exhibited activity of acetylcholiesterase but not of glyceraldehyde-3-phosphate dehydrogenase, the reverse being observed for the IO-oriented vesicles. The second phase of the sidedness experiments was related to the activity of Na^+-K^+- ATPase, a subject of intense research in Dr. Fortes' lab. The availability of a very sensitive biochemical method (Sanui, H. 1974. Anal. Biochem. 60: 489-504) applicable to the red cell plasma membrane, which exhibits a considerably lower Na^+-K^+- ATPase activity than rabbit kidney or eel electric organ, researched by other workers in the lab, stimulated interest to study the RO and IO vesicles particularly in relation to the action of ouabain, a powerful inhibitor acting on the external side of the membrane. Previous studies by other workers had dealt with intact red cells, sealed ghosts, or RO and IO vesicles employing radiovesicles, using red cell ghosts as controls, disclosed linear increments in activity of the enzyme, with little or no inhibition by ouabain, up to a period of 15 minutes after the addition of ATP, indicating an increase in the permeability of the vesicles as the period of incubation increased. Inhibition by ouabain ranged from 0 to 55 percent after 20 to 60 minutes and was comparable to that of the opened ghosts. Further characterization of the RO and IO vesicles was made employing a dimer of the fluorescent probe, 1-anilinonaphthalene-8-sulfonate, a compound known to inhibit the Na^+-K^+- ATPase, but whose penetration characteristics or membrane site of action is unknown. In the presence of magnesium ions this anionic probe exhibited a greater degree of fluorescence when exposed to IO vesicles, indicating a difference in its molecular environment. The fluorescent characteristics of these organic molecules are dependent upon their environment. Due to termination of the sabbatical period elucidation of the differences observed or of the degree of inhibition of this compound on the enzyme activity was not completed. However, similar challenging research of the plasma membrane of parasites e.g., Trypanosoma cruzi, should provide a greater

understanding of its structure and function and aid significantly in chemotherapeutic or immunologic studies.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z0I AI 00098-22 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.C. Weinbach	Head, Physiology and Biochemistry Section, LPD,NIAID
Other:	L.S. Diamond	Research Zoologist, LPD
	T. Takeuchi	Visiting Associate, LPD
	M. Gottlieb	Senior Staff Fellow, LPD
	C.E. Claggett	Biological Laboratory Technician (Biochem),LPD

COOPERATING UNITS (if any)
Clinical Haematology Branch, NIAMDD (C. Pazoles); Wenner Gren Institute, University of Stockholm, Stockholm, Sweden (T. Bernard).

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
35/12	23/12	12/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

As part of a broad program to study aerobic energy metabolism in parasites and mammals, current interest is focused primarily on the role of iron-sulfur flavoproteins as electron carriers in the respiratory chain of Entamoeba histolytica. Flavins were detected and analyzed by difference and fluorescent spectrophotometry; oxygen consumption was determined polarographically. Axenic amebae contained 7% Riboflavin, 21% FAD and 72% FMN of the total TCA-extractable flavins. The flavins, like non-heme iron, were found in the soluble fraction of E. histolytica. Riboflavin kinase was detected also in the soluble fraction. Its specific activity (4.6 Units/mg protein) approximated that of rat liver. In contrast to known kinases, the amebal enzyme was activated by Mn^{2+} instead of Mg^{2+}. These findings show that E. histolytica has the intrinsic capacity to synthesize flavonucleotides. A collaborative study of electron probe microanalysis of cestode calcareous corpuscles, initiated this year, showed differences in the distribution of metals in the complex organic-mineral structure of these granules. The collaborative studies on bovine adrenal medulla chromaffin granules disclosed that although uncouplers of oxidative phosphorylation

Summary of Work, cont'd: prevented the release reaction, presumably by discharging a proton gradient, anion channel blockers were ineffective as uncouplers, and must act by another mechanism. Catalase, but not peroxidase, was detected in the chromaffin granule fraction and may be involved in the synthesis of biogenic amines.

Project Description:

The object of this project is to conduct fundamental studies on the mechanisms of aerobic energy metabolism in mammalian and parasitic organisms. Currently, these studies center primarily on mechnisims of terminal electron transfer reactions and oxidative phosphorylation.

Tissues and cells are disrupted mechanically and subcellular fractions are isolated by differential and gradient centrifugations. Cellular and subcellular constituents, enzyme activities, and metabolic pathways are determined, characterized and elucidated by enzymatic, chemical, radiochemical and physical methods. Changes in oxygen concentration are determined polarographically with the Clark electrode. Electron probe microanalysis is used to localize elements in cestode calcareous corpuscles.

Parasite studies: Continued investigation of the respiratory chain in Entamoeba histolytica, strain HK-9, cultivated axenically in Diamond's liquid medium, was focused primarily on the iron-sulfur flavoproteins. The amebae contained 1% Riboflavin, 27% FAD and 72% FMN of the total TCA-extracted flavins. A highly unusual feature of the amebal flavin is that 98% was found in the soluble fraction of disrupted amebae. Only 2% remained bound to membranes; this closely corresponds to the 4% non-heme iron found in the membrane fraction (Claggett). These data, together with our previous finding that less than 1% of the oxidase activity is associated with amebal membrane, indicate that respiratory chain of E. histolytica, unlike that of aerobic bacteria or mammals, is soluble. Riboflavin kinase, which catalyses the synthesis of FMN, was found also in the soluble fraction. The specific activity of this enzyme (4.6 Units/mg protein) approximated that of rat liver. In contrast to other known kinases, the amebal enzyme was activated by Mn^{2+} instead of Mg^{2+} (Takeuchi). These data show that E. histolytica has the capacity to synthesize its own flavonucleotides, and may not require exogenous nucleotides for its survival. Multiple forms of NADPH diaphorase, detected by column chromatography, required flavonucleotides for their full activity. The enzymes were shown to be iron-sulfur flavoproteins (Takeuchi). Cestode calcareous corpuscles, previously shown to be composed of organic and irorganic constituents (largely complexes of Ca, Mg phosphates and carbonates) were examined by electron probe microanalysis. In addition to completely confirming our earlier analyses, the microprobe revealed an uneven distribution of metal ions in the corpuscles; there definitely is an outer shell and inner matrix of differing chemical composition (Barnard, Weinbach).

Mammalian studies: The study of bioenergetic phenomena in bovine adrenal gland chromaffin granules was continued in collaboration with Dr. Pazoles, NIAMDD. In contrast to last years findings that uncouplers of oxidative phosphorylation were potent inhibitors of the release reaction in granules, anion channel blockers which also block the release reaction, were ineffective as uncouplers with either intact mammalian mitochondria, or submitochondrial particles. The findings with uncouplers support the concept that electrochemical gradients are involved in the maintenance of granule integrity since these uncouplers destroy such gradients. Thus, anion channel blockers must inhibit by another mechanism (Weinbach). During these studies, we discovered that catalase, but not peroxidase, is associated with the granule fraction. Careful analysis of these fractions from sucrose density gradients (utilizing a novel polarographic method we developed to assay catalase activity in optically dense suspensions) showed another particle, isodense with chromaffin granules, may be the locus of catalase. It is not a typical peroxisome because other enzyme markers of that organelle were absent in the adrenal gland (Pazoles, Weinbach). The discovery of catalase in the adrenal gland chromaffin granules may have important physiological significance.

This investigation, which attempts to elucidate vital biochemical mechanisms common to all aerobic cells, has obvious implications for biomedical research in general (illustrated by the numerous collaborative projects either ongoing or planned), and provides a rational basis for understanding bioenergetic problems associated with parasites and parasitism.

The parasite studies will be continued with emphasis on whether ubiquinones function in electron transport in E. histolytica. Dr. Takeuchi, who returns to Japan in December, will complete his studies of the iron-sulfur flavoproteins; pyruvate and NADPH oxidases. As a result of our previous investigation on the metabolism of L-serine, we have initiated studies, in collaboration with Dr. Gottlieb, on the uptake and utilization of this and other amino acids by E. histolytica. This work, utilizing radiolabeled substrates, will attempt to determine the intracellular fate of the amino acids and the nature of the transport mechanisms. The electron probe microanalysis of cestode calcareous corpuscles, done in collaboration with Dr. Barnard, University of Stockholm, will be continued and perhaps completed. The mammalian work will be continued with primary emphasis on the intracellular localization and physiological role of catalase in the bovine adrenal gland and its association with chromaffin granules. In collaboration with Dr. R. Blaese, NCI, a study of energy metabolism in human blood platelets will be resumed. Dr. Blaese, who has been conducting immunological studies of patients with Wiskott-Aldrich syndrome, has suggested that impairment in the platelets energy metabolism may be implicated in this disease. The collaboration also will enable us to resume our studies on the NADPH diaphorase that we previously discovered in normal human platelets. The study of chemically-induced acute, intermittent porphyria in rats will be resumed in collaboration with Dr. D. Tschudy, NCI. Assessment of mitochondrial function in livers of these animals will be done initially.

Takeuchi, T., Weinbach, E.C. and Diamond, L.S.: Entamoeba histolytica: Localization and characterization of phosphorylase and particulate glycogen. Exp. Parasitol. 43: 107-114, 1977.

Takeuchi, T., Weinbach, E.C. and Diamond, L.S.: Entamoeba histolytica: Localization and characterization of phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase, and glycogen synthase. Exp. Parasitol. 43: 115-121, 1977.

Weinbach, E.C., Claggett, C.E., Takeuchi, T. and Diamond, L.S.: Biological oxidations and flavoprotein catalysis in Entamoeba histolytica. Arch. Invest. Med. (In press).

Takeuchi, T., Weinbach, E.C., Gottlieb, M. and Diamond, L.S.: Mechanism of L-serine metabolism in Entamoeba histolytica. Comp. Biochem. Physiol. B. Biochem. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00099-08 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Biophysical Parasitology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. A. Dvorak — Research Microbiologist, LPD, NIAID

Other: M. S. Crane — Research Associate, LPD, NIAID

G. A. Schmunis — Professor, UFRJ, Brazil

L. Koski — M.D., University of Maryland

COOPERATING UNITS (if any)
Applied Clinical Engineering Section, DRS; Television Engineering Section, CC; Instituto de Microbiologia, UFRJ, Brasil; Dept. of Neurology, Univ. of Maryland

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A multiparametric approach is being utilized to study the interaction of Trypanosoma cruzi with vertebrate cells. Vertebrate cell X T. cruzi hybrids have been produced which are stable and express parasite antigens. Changes in the intracellular cycle of T. cruzi have been quantified as well as the effects of immune human chagasic serum on the ability of the parasite to interact with human cells. The interference in penetration of vertebrate cells by T. cruzi trypomastigotes has been demonstrated using enzyme inhibitors. Reactivation of virulence has been achieved in the Ernestina strain of T. cruzi via a natural route following 14 years of continuous in vitro culture.

PHS-6040
(Rev. 10-76)

Project Description:

1. The production of vertebrate-parasite cell hybrids.

A great deal of research in recent years has been directed toward the production of hybridoma cell lines capable of expressing monospecific antibodies. Although cell hybrids of this type could be useful in studies of parasite-induced antibodies, the production of a vertebrate cell line capable of expressing parasite antigen has even more immediate and practical value. Dr. Crane has recently demonstrated that vertebrate-parasite cell hybrids can be produced. Using a modification of the polyethylene glycol fusion technique, Dr. Crane has succeeded in fusing T. cruzi epimastigotes to bovine embryo skeletal muscle cells and the continuous human cell line, Detroit-98. The Detroit 98 X T. cruzi hybrid cell lines appear to be stable and express T. cruzi antigen as determined by the ELISA technique. The morphologic events occuring during fusion have been documented.

Aside from the obvious usefulness of this system to basic cell biological questions, there are several major Chagas' disease-related problems that can now be studied. The parasite antigens can be isolated, characterized and used as specific immunogens. It is not yet known if all hybrid cell lines express the same antigens. If not, the cell hybrids could provide a model for the study of possible immunopathologic lesions resulting from T. cruzi infection.

2. Studies of the intracellular cycle of T. cruzi.

The major phases of the intracellular cycle of T. cruzi have been characterized using the steady-state culture system developed in the LPD. These data have been used as a base for studies of differences in the intracellular cycle of T. cruzi derived from various sources. The Y-strain of T. cruzi has been used for recent work. Because of its apparent increasing popularity as a laboratory research strain, it is a logical candidate for quantification of parameters of its intracellular cycle. The complete intracellular cycle of 200 individual parasites was quantified.

The first cycle doubling time and generation number in bovine embryo skeletal muscle cells of a population of mouse blood-derived parasites was significantly less than for parasites that had been continuously cycled for several weeks in cell culture. Consequently, the overall cycle time for the first cycle of blood-derived parasites is shorter than for parasites that had been acclimated to cell culture. This is apparently a general phenomenon as previous work by this laboratory has demonstrated a similar but even more marked lengthening of the intracellular cycle of T. cruzi in cell culture following passage from the insect host. The mean lag period prior to reproduction of Y-strain parasites is significantly shorter than with parasites of other

strains studied to date. Thus, the lag period may represent a parameter that is parasite strain specific and useful as a descriptor of the strain or as a means of classifying strains into groups. Both the lag and doubling times of the parasite populations are best described by normal distributions irrespective of passage history. These data indicate that although the intracellular cycle of T. cruzi can be cultured in vitro the culture conditions are sufficiently different from in vivo conditions to require a degree of accomodation by the parasite which is sufficiently severe to cause marked growth changes.

3. The influence of immune human serum on the ability of T. cruzi trypomastigotes to penetrate human cells.

The effects of acute and chronic human chagasic sera on the initial interaction of T. cruzi trypomastigotes was quantified in WI-38 human diploid fibroblasts. In the presence of acute sera, the attachment to or penetration of WI-38 cells by trypomastigotes was higher than but not significantly different from corresponding normal controls. However, in the presence of chronic sera the attachment to and penetration of WI-38 cells by trypomastigotes was significantly higher than normal controls. Adsorption of chronic sera with WI-38 cells significantly reduced the ability of trypomastigotes to attach to and penetrate WI-38 cells. Pre-incubation of trypomastogotes in chronic serum did not decrease their ability to penetrate WI-38 cells. Neither Fc nor C3 receptors were detected on the WI-38 cells. Trypomastigotes "capped" antibody in the presence of chronic but not acute serum. It is postulated that an antibody may be present in chronic human chagasic serum that reacts with both trypomastigotes and human cells and influences the ability of parasites to interact with human cells.

4. The effects of enzyme inhibitors on the penetration of vertebrate cells by T. cruzi trypomastigotes.

It has been reported by other workers that the pretreatment of vertebrate cells with proteolytic enzymes inhibits the ability of T. cruzi trypomastigotes to be taken up by the vertebrate cells. These data are used in an indirect argument to fortify the concept that phagocytosis is the sole mechanism of uptake of parasites by all vertebrate cells. Observations made in this laboratory do not agree with this concept and in attempts to repeat the experiments here, different results were obtained. The major problem proved to be the lack of a critical control in the proteolytic enzyme study reported by other workers.

It has now been established that some enzyme inhibitors decrease the ability of T. cruzi to enter vertebrate cells to a level that is statistically the same as occurs following enzyme treatment alone. These data can be correlated with as yet unpublished data from a German laboratory and surface interferometric data collected in the LPD to present a strong argument for active penetration of some vertebrate cells

by trypomastigotes *via* labile enzymes present on the surface of the parasite. The way is now open to begin studies directed at cell surface receptors which may also influence the interaction.

5. Reactivation of virulence in *T. cruzi*.

The Ernestina strain of *T. cruzi* has been passaged for about 14 years in cell and liquid medium culture by members of the LPD staff. Several attempts at passaging the strain back into animals were unsuccessful. Recently, the strain was successfully passaged into the insect *Dipetalogaster maximus*. Metacyclic trypomastigotes were obtained which were inoculated into mice. Acute lethal infections were produced in the mice using small inocula. Blood-derived trypomastigotes can be passaged to mice producing lethal infections. The previously reported protocols for this type of work utilizing high temperature or x-irradiation were not employed here. Consequently, the reactivation of virulence was naturally induced and not the result of a potentially mutagenic procedure. Physiological and cell biological parameters are now being compared in the insect-derived, blood-derived and tissue culture-derived material.

Significance and Proposed Course of Program:

(1) The study of host-parasite interactions at the cellular and subcellular level is a topic of critical importance. The outcome of this interaction directly influences the physiologic state and survival potential of both the host and the parasite.

(2) The methodology and research philosophy being developed are directly applicable to other areas of biomedical research. For example, the controlled-environment culture system developed with this program is now in routine use in laboratories with such diverse interests as oncology, immunology, neurophysiology and morphogenesis. The video systems developed with this program are being used for analysis of macroscopic as well as microscopic images in basic research and diagnostic clinical-medical fields.

Research will continue in an attempt to further our understanding of the interaction of *T. cruzi* with vertebrate cells *in vitro* and relate this information to the course of Chagas' disease in nature. Basic information obtained with the controlled-environment culture system and video systems will be utilized in more complex *in vitro* model systems.

The basic methodology and research philosophy developed with this program will be used for the analysis of other host-parasite interactions.

Publications:

1. Dvorak, J. A. and Howe, C. L.: *Toxoplasma gondii*-vertebrate cell interactions: I. The influence of bicarbonate ion, CO_2, pH and host cell age on the invasion of vertebrate cells *in vitro*. *J. Protozool.*

24: 416-419, 1977.

2. Dvorak, J. A. and Howe, C. L.: Toxoplasma gondii-vertebrate cell interactions: II. The intracellular reproductive phase. J. Protozool. (in press).

3. Weller, P. F., Dvorak, J. A. and Whitehouse, W. C.: Human eosinophil stimulation promoter lymphokine: Production by antigen stimulated lymphocytes and assay with a new electro-optical technique. Cellular Immunol. (in press).

4. Dvorak, J. A.: Host-parasite relationships at the cellular level in Trypanosoma cruzi infections. in Chagas' Disease, proceeding of an international symposium held in conjunction with the Fifth International Congress on Protozoology. PAHO Scientific Publication No. 347 pp. 1-10, 1977.

5. Mason, S. J., Miller, L. H., Shiroishi, T., Dvorak, J. A., and McGinniss, M. H., The duffy blood group determinants: Their role in the susceptibility of human and animal erythrocytes to Plasmodium knowlesi malaria. British J. Hematol. 36: 327-335, 1977.

6. Dvorak, J. A. and Schunis, G. A.: The influence of acute and chronic human chagasic sera on the ability of Trypanosoma cruzi to attach to and penetrate human diploid fibroblasts (abs, 4th Int. Congress of Parasitol., in press).

7. Miller, L. H., Mason, S. J., Dvorak, J. A., Shiroishi, T. and McGinness, M.: Erythrocyte receptors for malaria merozoites and the duffy blood group system in Human Blood Groups, 5th Int. Convoc. Immunol., Buffalo, N. Y. 1976 pp. 394-400.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00101-04 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Characterization of Polysaccharides in protozoa of the family Trypanosomatidae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. Gottlieb, Senior Staff Fellow

Other: E.C. Weinbach, Head, Section on Physiology and Biochemistry, LPD/NIAID

COOPERATING UNITS (if any)
Department of Biology, Johns Hopkins University (Drs. Roseman and Meadow)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

INSTITUTE AND LOCATION
NIAID, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
22/10	10/12	12/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Polysaccharides and carbohydrate-containing components of various hemoflagellates of the parasitic family Trypanosomatidae have been studied. Polysaccharides isolated from a number of species of the genus Crithidia have been separated and characterized as previously reported for Crithidia fasciculata. Relationships between the organisms within this genus have been established by chemical analysis of these constituents. A circulating antigen from mice infected with Trypanosoma cruzi, previously identified with a carbohydrate containing antigen extracted from culture forms (epimastigotes) of Trypanosoma cruzi, has been quantitated and shown to be proportional to the parasitemia in the mouse. Evidence for the loss or shedding of this antigen from trypanosomes in vitro has so far been negative. Hexose and glycerol uptake by Leishmania tropica have been investigated. Sugar and glycerol phosphorylating activities were characterized with respect to several kinetic parameters, substrate specificity, cofactor requirements and effects of inhibitors. The results indicate a very close relationship between transport and the phosphorylation step. The uptake and utilization of

Summary (Continued)

amino acids and other metabolites by Entamoeba histolytica is being studied. The intracellular fate of these substrates is being determined.

Project Description:

The studies described were continued in an effort to chemically and biochemically characterize polysaccharide constituents of hemoflagellates (protozoa of the family Trypanosomatidae). As a result of our findings these studies were broadened to include analysis of the role of these compounds in the parasitic process as well as studies on the relationship of these constituents to the cell membrane and surface of these parasites.

Studies on the functional aspects of the cell membrane were also carried out. These investigations focused on metabolite transport (primarily carbohydrates) in Leishmania tropica promastigotes (culture forms). Recently, these studies have been extended to include examination of amino acid transport and utilization in Entamoeba histolytica.

Polysaccharide Studies. Crithidia fasciculata and other Crithidia spp: Protozoa of the genus Crithidia are monoxenic trypanosomatids, parasitic in insects, which have been used as models for the pathogenic members of the family. These organisms are morphologically indistinguishable.

Phenol-water extracts of Crithidia fasciculata has been shown to contain two polysaccharides--a mannan and an arabinogalactan which has been localized at the cell surface. The polysaccharides are separable by column chromatographic techniques. Similar extractions and separations have been carried out on seven other members of the genus Crithidia. The results of polysaccharide analysis suggest certain relationships within the group which correlate with insect order from which the flagellate was isolated. Comparisons have also been made on the basis of agglutinability by specific antibody and lectins specific for surface carbohydrate moieties.

Investigations on the biosynthesis of polysaccharide constituents have also proceeded. The incorporation of 2 deoxy glucose into the phenol-water extracted polysaccharides of C. fasciculata has been monitored using the radiolabeled compound.

Trypanosoma cruzi. Quantitative studies on the circulating carbohydrate-containing antigen in T. cruzi infected mice has been carried out. The results of a rocket immunoelectrophoretic technique demonstrate the positive correlation between the quantity of circulating parasite antigen and the parasitemia.

Results, to date, in vitro have failed to provide evidence for the release of the carbohydrate containing antigen in either cell free cultures of epimastigotes or in tissue cultures infected with T. cruzi.

Project Description (Continued)

Although a large number of human sera have been tested, only a small number of these have been shown to contain antibodies to the circulating carbohydrate antigen by precipitin methods. The few sera in which antibodies were detected were from acutely infected individuals. In one case, the rise and fall in antibody titer was followed for more than one year of infection.

Metabolite Transport and Utilization Studies:

Leishmania tropica. The transport and subsequent metabolism of sugars by L. tropica has been studied. The uptake and concentration of sugars (and glycerol) from the media into intact promastigotes (culture forms) has been followed by the use of radiolabelled sugars. Analysis of intracellular enzymatic activities has been carried out on cell extracts and homogenates.

The results indicate that the first detectable intracellular product of transport is a phosphorylated form, presumably hexose-6-phosphate. Kinetic analysis has not allowed us to distinguish between the two following models: 1) transport of the free sugar followed by a hexokinase type enzyme or 2) phosphorylation of the sugar concomitant with transport (cf. bacterial phosphotransferase system).

Analysis of the phosphorylation (hexokinase) activity has revealed the following information:

1) The enzyme is primarily particulate and is not solubilized by Triton X-100 or high salt treatments.

2) The enzyme does not appear to concentrate with a plasma membrane fraction.

3) The sugar specificity of the enzyme is identical to the specificity of the transport phenomenon.

4) The enzymatic activity has been characterized in terms of phosphoryl donor (nucleoside triphosphates not phosphoenol-pyruvate), temperature optimum, divalent cation requirements and other kinetic parameters.

5) The enzymatic activity is relatively heat stable and refractory to sulfhydryl reagents.

A glycerolkinase and glycerol transport system has also been identified and distinguished from the hexose system described above based on competition and sulfhydryl reagent studies.

Several other enzymes of glucose metabolism have been identified and characterized in terms of intracellular distribution.

Project Description (Continued)

The intracellular fate of 2 deoxy D-glucose, "a non-metabolizable" analog of glucose, has been followed.

Entamoeba histolytica: As a result of studies on the oxidation of serine by the amoebae, we have undertaken (with Dr. Weinbach) an examination of the transport and utilization of this and other amino acids and metabolites by this organism. Transport is studied by uptake of labeled metabolite. The subsequent metabolism of the substrate is followed into various intracellular pools by various precipitation and extraction techniques. Among the amino acids tested particular attention is given to cysteine which is extremely important in the maintenance of the amoebae in both growth and stasis media.

These studies, on the functional as well as compositional aspects of the cell surface and membranes coupled with further studies on metabolism of these parasites, will lead to a better understanding of the nature of parasitism and survival in these protozoa.

This project was terminated as of July 29 because the principal investigator resigned to become an Assistant Professor in the Department of Pathobiology at Johns Hopkins University School of Hygiene and Public Health. However, work with the amoebae will continue on a collaborative basis with Dr. Weinbach.

Publications:

Gottlieb, M.: A carbohydrate-containing antigen from Trypanosoma cruzi and its detection in the circulation of infected mice. J. Immunol. 119: 465-470 (1977).

Gottlieb, M.: Polysaccharides of Crithidia fasciculata. Identification and partial characterization of a cell surface constituent. Biochem. Biophys. Acta., (in press).

Gottlieb, M.: Trypanosoma cruzi: Identification of a cell surface polysaccharide. Exptl. Parasit., (in press).

Takeuchi, T., Weinbach, E.C., Gottlieb, M. and Diamond, L.S.: Mechanism of L-serine oxidation in Entamoeba histolytica. Comp. Biochem. Physiol., (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00102-04 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Pathogenesis of Disease Caused by Infection with Intracellular Parasites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: F.A. Neva — Chief, Laboratory of Parasitic Diseases, NIAID

Others: Renato Gusmão — Visiting Fellow, LPD,NIAID
Albert Gam — Biological Laboratory Technician (Micro.), LPD,NIAID

COOPERATING UNITS (if any) Professors Joffre Rezende and Anis Rassi, University of Goias, Goiania, Brazil. Professor Aluizio Prata, University of Brasilia, Brasilia, Brazil. Dr. Frances Ward, Duke University Medical Center, Durham, N.C. Dr. Euripides Ferreria, University of Parana, Curtiba, Brazil.(continued)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Cell Biology and Immunology Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
27/12	16/12	11/12

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lymphocyte blastogenesis to various T. cruzi antigens was tested in patients with chronic Chagas' disease and found to be present but variable in degree. Magnitude of response of this test of cell-mediated immunity showed no obvious correlation with clinical features of disease. During the same study the frequency of HLA tissue types in Chagas' patients was compared to appropriate controls. A reduced frequency of one antigen type at the A locus and possible increased frequency of two antigenic types, one at the A and the other at the B locus, was found in patients with chronic Chagas' disease.

A plaquing technique for quantitative estimation of minimally infective units of T. cruzi for cells has been developed. This technique appears applicable to testing strains of T. cruzi directly from infected bugs.

The genetic pattern of susceptibility to a strain of Leishmania tropica in mice suggests control by a single gene.

PHS-6040
(Rev. 10-76)

Cooperating Units, cont'd:

Dr. Jonathan Weintraub (former Research Associate) Stanford University, CA. Dr. David H. Sachs, Immunology Branch, NCI, NIAID and Dr. David Alling, NCI.

Project Description:

Immunologic studies with T. cruzi, the causative agent of Chagas' disease, were directed almost entirely to comparative assessment of lymphocyte blastogenesis to various T. cruzi antigens in patients with Chagas' disease and appropriate controls. Such a project required an unusual degree of cooperation with several groups of Brazilian collaborators and colleagues for patient material as well as laboratory facilities. Since such well selected patient material was available to us the project was expanded to include tissue typing of patients with Chagas' disease and appropriate controls to seek clues over possible genetic influence in development of chronic Chagas' disease. Lymphocyte blastogenesis tested by thymidine incorporation with variable doses of five different specific antigens, several unrelated antigens and mitogens in a total of 24 patients and 19 controls was made possible by the multi-sample automated cell harvester. The HLA-tissue typing was done with the collaboration of former and present personnel of the tissue-typing laboratory from Duke University using reagents developed through support of the NIAID Extramural Immunology Branch. Results of the lymphocyte reactivity tests showed that this measure of cell-mediated immunity was intact but variable in degree in chronic cases with both cardiomyopathy as well as mega-esophagus. Very high reactivity had no correlation with clinical features of disease. The amastigote-trypomastigote antigen prepared from T. cruzi infected cell cultures gave overall best responses but several crude epimastigote antigens also gave perfectly suitable blastogenic responses. (Neva, Gusmão, Gam and collaborators).

The HLA-typing part of the Brazilian study have disclosed a negative and positive correlation of certain HLA types in chronic Chagas' disease. The negative correlation involving one type at the A locus was found in 34 patients with chronic heart and mega disease, but was most striking in the latter group of 19 cases. A weakly positive correlation, or increased frequency of cases compared to controls, occurred with one type at the A locus and one type at the B locus. The association of certain tissue types with various diseases is currently a subject of intense interest and a linkage has never yet been established with a parasitic disease. In view of the many unknown factors in pathogenesis of chronic Chagas' disease this clue as to genetic influence seems to warrant further study of the relationship, so an expanded project is planned for next year. (Neva, Gusmão and collaborators).

For the assessment of in vitro infectivity for cells of different strains of T. cruzi greatest emphasis was given to the plaquing technique. A linear dose-response relationship with the cell culture adapted strain (Ernestina) was found and this will be sought using strains of T. cruzi directly from infected arthropod vectors. Use of the plaquing technique to demonstrate modification or selection of populations of T. cruzi has been

initiated, in this case with the Ernestina strain grown at high and low temperature (33° vs 38°C). The optimal conditions required for plaque formation with T. cruzi remains a problem and does not yield as rapid results as with phage or mammalian viruses. However, the focus on technical requirements has yielded a by-product type of finding concerning CO_2 enhancement for multiplication in the physiology of T. cruzi. CO_2 was found necessary for plaque formation but also enhanced or accelerated trypomastigote output from infected cells. (Neva and Gam).

Work on the experimental mouse model for Leishmania tropica infection involved seeking evidence for a genetic influence on susceptibility and resistance to infection with in-bred strains of mice. Since C57 Bl and DBA mice are resistant to the "S" strain L. tropica it was possible to determine that susceptibility was not related to the H2 tissue type nor to immunoglobulin allotypes. However, the frequency of foot pad lesions observed in the backcross with the susceptible parental strain was suggestive of a single gene inheritance pattern for susceptibility. (Neva and cooperating units).

Publications:

Neva, F.A.: Looking back for a view of the future: Observations on immunity to induced malaria. Am. J. Trop. Med. Hyg. 26: 211-215-,1977.

Weintraub, J., and Weinbaum, F.I.: The effect of BCG on experimental cutaneous leishmaniasis in mice. J. of Immunol. 118: 2288-2290, 1977.

Neva, F.A., and Ottesen, E.A.: Current concepts in parasitology: Tropical (Filarial) Eosinophilia. New Eng. J. of Med. 298: 1129-1131, 1978.

Miller, L.H., Neva, F.A., and Gill, F.: Failure of chloroquine in human Babesiosia (Babesia microti) case report and chemotherapeutic trials in hamsters. Ann. of Int. Med. 88: 200-202, 1978.

Ottesen, E.A., Hiatt, R.A., Cheever, A.W., Sotomayor, R., and Neva, F.A.: The acquisition and loss of antigen-specific cellular immune responsiveness in acute and chronic schistosomiasis in man. Clin. Exp. Immunol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00103-11 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Immunological Studies on Toxoplasmosis and Other Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.N. Lunde	Research Zoologist
Other:	H.G. Sheffield	Head, Cell Biology & Immunology Section, LPD, NIAID
	A.W. Cheever	Assistant Chief, Laboratory of Parasitic Diseases, and Head, Host-Parasite Relations Section
	E.A. Ottesen	Medical Officer, LPD
	T.E. Nash	Medical Officer, LPD
	A.A. Gam	Biological Laboratory Technician (Micro.), LPD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Cell Biology and Immunology

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
18/12	13/12	5/12

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
This study was initiated to improve ELISA techniques for serodiagnostic use in parasitic diseases. Sera from patients with well defined acute and chronic schistosomiasis were examined by ELISA using egg, cercarial and adult worm antigens. Sera from acute schistosomiasis patients had higher titers with cercarial than adult worm antigen while patients with chronic schistosomiasis had higher titers with adult worm antigen. We were thus able to distinguish sera from patients with well defined acute and chronic schistosomiasis.

PHS-6040
(Rev. 10-76)

Project Description:

Emphasis has been focused on improving enzyme-linked immunosorbent assay (ELISA) for serodiagnosis and study of host parasite immune responses in parasitic diseases, especially schistosomiasis. The following findings relative to schistosomiasis were established:

1. Sera from patients with acute schistosomiasis (<2 years duration) were found to have higher ELISA titers with cercarial antigen than with adult antigen. In contrast sera from patients with chronic schistosomiasis (>2 years duration) were found to have higher titers with adult worm antigen than with cercarial antigen. This difference could be expressed and quantified by determing the ratio of cercaria/adult (c/a) antigen $OD_{490\ nm}$ at a 1:16 dilution of serum. Acute infections had high c/a ratios (1.7 ± 037) while chronic infections had low c/a ratios (0.74 ± 0.26). We were thus able to distinguish sera from patients with well defined acute and chronic schistosomiasis very effectively.

2. Acute patients, those with high c/a $OD_{490\ nm}$ ratios, had schistosome specific IgM antibody demonstrated by ELISA.

3. Schistosome specific IgE antibody was demonstrated by ELISA in patients with acute schistosomiasis. This antibody response was to soluble egg antigen.

4. Re-exposure of chronically infected monkeys to infected cercariae failed to alter an already established low c/a ratio.

5. There was no evident relationship between serological response, egg and adult worm burden in monkeys. Likewise there was no correlation between egg excretion and c/a ratios in the human cases studied.

Proposed Course:

1. The studies with schistosomiasis will be continual and expanded to cover circulating and purified antigens. (Nash and Boctor). More information concerning specific IgE, IgA and IgM response will be sought.

2. Other parasitic diseases will be added for ELISA evaluation as time and available collaborators permit. Preliminary studies are currently in progress with trypansomiasis and strongyloidiasis. (Gusmao and Gam).

Significance to Biomedical Research:

Application of ELISA for multipurpose seroepidemiological and clinical studies is being contemplated in areas endemic for malaria, trypanosomiasis and schistosomiasis by the World Health Organization. We have just shown how ELISA using both cercarial and adult antigens can be a useful technique in distinguishing acute and chronic infections thus making it useful for seroepidemiological and clinical studies. Further investigations employing ELISA with specific and purified antigen should make serology a more useful

tool and make interpreting of results less difficult.

Publications:

Stalheim, O.H., Proctor, S.J., Fayer, R., and Lunde, M.N.: Death and Abortion in Cows Experimentally Infected with Sarcocystis from Dogs. 19th Annual Proc. Am. Assoc. Vet. Lab. Diagnosticians. 1976: 317-329.

Lunde, M.N., Ottesen, E.A., and Cheever, A.W.: Serological Differences Between Acute and Chronic Schistosomiasis Mansoni Using Enzyme-Linked Immunosorbent Assay (ELISA). Am. J. Trop. Med. Hyg. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00104-04 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Structure and Development of Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H.G. Sheffield Head, Section on Cell Biology and Immunology LPD NIAID

COOPERATING UNITS (if any)
Animal Parasitology Institute, ARS-USDA, Beltsville, Maryland

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Cell Biology and Immunology

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
24/12	12/12	12/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the cell biology of various parasites in relation to the organism itself, its interaction with the host cell and production of disease. Current areas of interest are: 1) development of immunity: 2) effect of drugs on the parasite life cycle; 3) comparative ultrastructure of infective forms (sporozoites) of related parasites; and 4) asexual and sexual development of the parasite. Emphasis is placed on Toxoplasma gondii and toxoplasmosis with members of the genera Hammondia, Sarcocystis and Isospora being used for comparative purposes. Techniques used include electron microscopy, tissue culture, serology and animal infections.

PHS-6040
(Rev. 10-76)

Project Description:

Cellular structure and changes related to growth, differentiation and multiplication of parasitic protozoa and response of host tissue to parasitic infection are studied. Aspects of infection and disease related to structure and development, particularly in toxoplasmosis, are examined.

Immunological response in relation to prevention of reinfection is studied in toxoplasma-infected cats. In addition to standard parasitological techniques, in vitro cultivation and electron microscopy are utilized.

Last year attempts were made to determine the cause of an outbreak of toxoplasmosis at a NIH contract sheep-breeding farm. High antitoxoplasma antibody titers in cats on the farm suggested that the cats were the source of the outbreak. Continued serological study of the sheep and removal of the cats has been accompanied by absence of seroconversion, abortion or evident toxoplasmosis. (Lunde, Z01 AI 00103-11 LPD). Laboratory experiments were initiated to determine the possibility of previously infected cats reexcreting oocysts of Toxoplasma gondii following repeated ingestion of toxoplasma-containing flesh. Cats challenged 17 weeks after a primary infection did not excrete oocysts. This finding confirms previous laboratory work. Studies utilizing frequent challenge and examination of feces for oocysts have not been completed.

Development of Sarcocystis cruzi in dogs has been studied as in previous years. Stages of oocyst (sporocyst) development were observed in the intestinal epithelium of dogs at various times after infection. The earliest stages were seen 1 day after infection. Attempts were then made to find the sexual stages preceding oocyst formation. Organisms digested by pepsin and trypsin from cysts in beef heart muscle were inoculated directly into the lumen of the small intestine of dogs after ligation of a 100cm section about 250 cm from the pylorus. Specimens for examination by electron microscopy were taken by biopsy or autopsy at times up to 24 hours after infection. No parasites were found in the intestinal tissue suggesting that the inocula were not infective or that the parasites need to be preconditioned in some way while passing through the stomach.

Heavy infections in dogs were produced using bovine heart muscle containing large numbers of cysts of S. cruzi. Samples were taken on autopsy at 1 to 24 hours after feeding the infected muscle to the dogs. Early stages of infection were seen at 1 hour after infection. Elongate parasites resembling the cyst organisms were found in the cytoplasm of mucous cells in the small intestine. Comparative studies suggest that after penetration of the mucous cell the organism becomes rounded and transforms into either a micro- or macrogametocyte. Macrogametocytes can be recognized by enlargement of the nucleus and separation of chromatin masses. Subsequently, the nucleus divides into several nuclei which migrate to the periphery of the microgametocyte. Flagellae (two per microgamete) form at the surface prior to budding off of the microgametes. Microgametogenesis is complete in 6 to 12 hours after infection.

Maturation of macrogametocytes into macrogametes parallels microgametogenesis. Several layers, reported last year, form around the macrogamete in the mucous cell. The outermost layer is a product of the mucous cell. Next is a membrane of host cell origin which borders the parasitophorous vacuole. The macrogamete itself is bounded by a loosely fitting membrane which appears to be cast from the parasite. The cytoplasm of the macrogamete is bounded by the usual two layered pellicle. Data suggest that these layers are formed prior to fertilization of the macrogamete. By 24 hours after infection, the macrogametes are found in the intercellular space of the lamina propria. All layers including the outer layer formed by the mucous cell remain around the macrogamete. No other trace of the mucous cell remains. It is not known whether the macrogamete migrates prior to fertilization or afterwards as a zygote.

The most significant finding is the first definitive observation of fertilization in the coccidia. Fusion of the membranes of the microgamete and macrogamete occurs followed by transfer of the chromatin portion of the microgamete nucleus into the cytoplasm of the macrogamete. Fusion of the chromatin with the nucleus of the macrogamete has not been observed. The remaining organelles of the microgamete do not enter the macrogamete. At the time of fertilization no flagellae were seen; their loss may occur following penetration of the microgamete into the parasitophorous vacuole. Stages in the development of the oocyst sporocyst wall within the layers surrounding the zygote have been observed within the first 48 hours after infection.

This project will be terminated August 1, 1978 upon transfer of the principle investigator from the Laboratory.

Publications:

Sheffield, H. G., Frenkel, J. K., Ruiz, A.: Ultrastructure of the cyst of Sarcocystis muris. J. Parasitol. 63: 629-641. 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00108-07 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies on the Biology and Immunogenicity of Malarial Sporozoites.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. W. Gwadz Research Entomologist, LPD, NIAID

Other: D. H. Chen Staff Fellow, LPD, NIAID

COOPERATING UNITS (if any)
New York University, School of Medicine, N.Y., N.Y., (R. Nussenzweig)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
18/12	3/12	15/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study the immune mechanisms involved in sporozoite-induced infections in simian, Plasmodium knowlesi, malaria.

Studies underway are concerned with development of methods for immunization of monkeys with P. knowlesi sporozoites.

PHS-6040
(Rev. 10-76)

Sporozoite Immunization:

Several rhesus monkeys have now been immunized with I.V. injections of radiation attenuated sporozoites and have survived repeated challenge. Monkeys receiving similar doses of sporozoites emulsified in Freund's complete adjuvant and administered I.M. were not protected.

Circum-sporozoite precipitin antibodies developed in monkeys after both types of immunization; the CSP reaction in I.V. injected monkeys was strong and persistant while the reaction in I.M. injected monkeys was weak and transcient.

Proposed Course:

The following investigations will be pursued in the coming year with the eventual goal of immunization with sporozoites of the human malarias. 1) improvement of the P. knowlesi/monkey model with respect to immunizatio schemes; 2) development of an alternative to I.V. inoculation; 3) improvement of sporozoite harvesting techniques and development of methods for infecting mosquitoes on in vitro cultured gametocytes.

Significance:

A sporozoite-based vaccine capable of preventing malaria infections would be of great importance to human populations in much of the tropical world. The ready application of this type of immunization has met with a number of practical difficulties. The development of a simian model for studies of the mechanisms and methodology for sporozoite-induced immunity would be very useful for the solution of some of these problems.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00109-06 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cellular Immunology of Malaria and Other Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D.J. Wyler, Senior Investigator, LPD, NIAID

Other: C.N. Oster, Senior Staff Fellow, LPD, NIAID
T.C. Quinn, Research Associate
J.D. Berman, Clinical Associate, LCI, NIAID
L.M. Wahl, Senior Staff Fellow, LMI, NIDR
S.M. Wahl, Research Microbiologist, LMI, NIDR
A.W. Cheever, Assistant Chief, LPD, NIAID

COOPERATING UNITS (if any)

Laboratory of Microbiology and Immunology, NIDR

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
24/12	11/12	13/12

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Investigations are directed at host defense mechanisms and immunoregulation in malaria and leishmaniasis, and the possible role of the schistosomal egg granuloma in regulation of hepatic fibrosis. Presently, research is aimed at (1) spleen function in malaria immunity (2) regulation of intracellular growth of leishmania in human macrophages, and (3) characterization of a fibroblast stimulating factor produced in vitro by schistosomal egg granulomas. In addition, clinical studies are covered by project Z01 AI 00141-02 FY 1978.

PHS-6040
(Rev. 10-76)

Project Description:

This project is aimed at the investigation of cellular immune mechanisms in parasitic diseases, with particular reference to protozoal infections but including interests in the immunopathogenesis of hepatic fibrosis in schistosomiasis. The studies are motivated by two concerns: (1) what are the critical issues of immune response in the particular parasitic disease with respect to host defense or pathophysiology?; and (2) in what way might these diseases serve as models to investigate aspects of host defense or pathophysiology which might also be important in other infectious or inflammatory diseases?

The major investigative efforts are directed at malaria immunology. The need for a greater understanding of host defense mechanisms and immune regulation in this disease are dictated by hopes for an eventual anti-malarial vaccine, coupled with the limitations of empiric approaches so far developed. The central role of the spleen in malaria host defense has stimulated investigations of the role of this organ in the intravascular clearance of parasitized erythrocytes in immune animals, and efforts at identifying spleen cell populations responsible for mediating host defense and immunosuppression. We have obtained evidence that the intravascular clearance of parasites depends upon the interaction of infected erythrocytes with the spleen on the basis of rheologic rather than immunologic factors (Quinn and Wyler). Since reconstitution with autochthanous spleen cell suspension renders splenectomized mice capable of resolving their malaria infections, whereas non reconstituted mice die (Oster and Wyler), we have established an in vivo model with which to study the role of specific spleen cell populations in host defense. Alterations in macrophage-mediated immunoregulation in malaria have been found to be attributable to the direct interaction between infected erythrocyte and spleen macrophages (Wyler)

Studies are being carried out in an effort to explore the interface between T cell activation and macrophage-mediated intracellular killing, using leishmaniasis as a model. This disease is associated with evasion of cellular immune mechanisms by the parasite. Infection with leishmania of human monocyte-derived macrophages has been achieved in vitro, and efforts are being directed at determining growth requirements of these cells (Wyler and Berman). This in vitro system will interdigitate with clinical studies (Project AI-00-141-02, FY 1978).

The relationship between delayed hypersensitivity and subsequent fibrosis is being studied in a murine model of schistosomiasis (Wyler, Wahl, Wahl, Cheever). We have determined that isolated schistosomal egg granulomas secrete substances in vitro which can stimulate fibroblast proliferation and collagen synthesis. These observations are the first direct evidence of a molecular link between chronic inflammation and subsequent fibrosis.

Publications

Wyler, D.J., and Brown, J.: Malaria antigen-specific T cell responsiveness during infection with Plasmodium falciparum. Clin. Exp. Immunol. 29: 401-407, 1977.

Wyler, D.J., Wahl, S.M., Wahl, L.M.: Hepatic fibrosis in schistosomiasis. Egg granulomas secrete fibroblast stimulating factor in vitro. Science. (In press).

Wyler, D.J.: Leishmaniasis in Current Therapy, 1979 (H.F. Conn, ed.)

Wyler, D.J. and Miller, L.H.: Malaria in Principles and Practices of Infectious Diseases (G. Douglas, G. Mandell, J. Bennett, eds.). (In press).

Wyler, D.J., Oster, C.N., and Quinn, T.C.: The role of the spleen in malaria infections. Bull. W.H.O. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00149-03 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Physiology of Anopheles mosquitoes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. F. Beach	Staff Fellow, LPD, NIAID
	R. N. Rosenberg	Staff Fellow, LPD, NIAID
Others:	R. W. Gwadz	Research Entomologist, LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
40/12	20/12	20/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project involves physiological studies on the Anopheline mosquitoes which serve as vectors of malaria. Two lines of investigation are being pursued at present: (1) Investigation of the neuro-hormonal control of blood feeding behavior. (2) The physiological control of egg laying behavior.

PHS-6040
(Rev. 10-76)

Project Description:

The objective of this project is to understand the physiological factors which control blood feeding behavior (and therefore disease transmission) in mosquitoes.

Neuro-Hormonal Control of Blood Feeding Behavior:

Once a female mosquito has mated she will spend the remainder of her adult life alternating between two distinct behaviors: blood feeding and the laying of eggs produced from this blood. It is because she repeats these behaviors that the female is such an efficient vector of disease causing organisms.

Following each blood meal the female mosquito enters a 2 to 3 day period when she no longer shows any interest in taking a blood meal. Such females ignore a human hand or other potential blood meal source until the previous meal is converted into eggs. Then, she again displays aggressive biting behavior at any opportunity.

Females whose ovaries have been surgically removed do not show this period of abstinence following a blood meal and will return to feed on subsequent days. Thus the developing ovaries are some how involved in turning-off blood feeding behavior.

The developing ovary is known to act as an endocrine gland secreting the insect steroid hormone ecdysone. Injections of this hormone into normal females suppresses their interest in taking a blood meal. This result suggests that the rise in ecdysone titer caused by the developing ovary also acts as a physiological trigger turning off biting behavior.

The Physiological Control of Oviposition:

Female mosquitoes will not begin oviposition unless the male pheromone matrone and mature eggs are present. However, when the ventral nerve cord of a mosquito is surgically cut she goes thru oviposition behavior even if the matrone stimulus and mature eggs are not present.

These experiments indicate that oviposition is normally inhibited through a negative feedback system centered in the ventral nerve cord of the animal. A matrone stimulus plus a similar input from the egg laden ovary turn off this inhibitory pathway thereby permitting the females to engage in oviposition. Cutting the ventral nerve cord mimics these events by abolishing this inhibitory nervous connection.

We are currently investigating how the matrone and egg stimuli reach the ventral nerve cord and how the negative feed back system can be short circuited by the use of drugs which are known to block inhibitory neuro transmitters in insects.

Proposed Course:

The following investigations will be pursued in the coming year: 1) At attempt to block blood-feeding behavior in malaria mosquitoes by feeding them ecdysone. If the hormone can be fed to mosquitoes and does inhibit blood feeding behavior then a means of incorporating it into their natural foods (nectars) will be investigated. 2) Demonstration of how oviposition behavior is turned on and off and determination of how this behavior can be most effectively inhibited.

Significance to Biomedical Research:

Anopheline mosquitoes are wholly responsible for the transmission of malaria from man to man. Their physiology is poorly understood. Our investigations are aimed at understanding certain critical and possibly vunerable stages in the life cycle of these disease vectors. Disruption of one of these events could provide a means of controlling the vector species.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00160-02 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies on the Cell Biology of E. histolytica and other Species of Entamoeba

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Frances D. Gillin Senior Staff Fellow, LPD, NIAID

Other: Louis S. Diamond Head: Parasite Growth and Differentiation Section, LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Parasite Growth and Differentiation

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
18/12	12/12	6/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

I Entamoeba: The temperature ranges and optima for axenic growth of E. invadens 165 and E. histolytica were determined. Trophozoites were rapidly killed by exposure to temperatures 2-3° above the maximum growth temperature, but killed very slowly at 0°. Metabolically active E. histolytica cells were much more sensitive to killing by oxygen than cells at 0°. Studies on the mechanism of attachment, motility and endocytosis have continued, with particular interest in the role of cysteine and ascorbic acid which cannot be replaced by other reducing agents or N_2 in the defined composition "Motility Buffer" we formulated.

II Giardia lamblia: Studies on this common intestinal protozoan parasite have recently been initiated: attached and swimming trophozoites have been separated and their axenic growth and attachment characteristics are being determined. Our observation that L-cysteine is specifically required for cellular integrity, attachment and growth is being pursued. We have started

PHS-6040
(Rev. 10-76)

Summary (Continued)

studies on the cell surfaces of G. lamblia trophozoites and cysts using fluorescent and enzymatic probes.

Project Description:

I Sensitivity of Entamoeba Trophozoites to Heat, Cold, and Air.

Only the motile trophozoite form of E. histolytica which is the invasive form in vivo, has been grown in axenic culture. It has long been observed that trophozoites were very delicate and were killed by exposure to non-optimal temperatures or oxygen concentrations. However, this has been difficult to quantitate. In the last report, we described a new method for clonal growth of E. histolytica and reptilian Entamoeba in agar. This method has provided a valuable assay for viability. Prior to determining the thermal sensitivity of these Entamoeba, we found the temperature ranges for growth (both in liquid medium and as clones in agar) to be 32 to 40 C for E. histolytica. The optimum was 35 to 37 C. The range for E. invadens 165 was 15 to 30 C, optimum 24 to 27 C. The lowest temperature for thermal killing of E. histolytica was 41.5 C and for E. invadens 35.5 C. Both were killed very rapidly at 42 C, but very slowly at 0 C. This resistance to cold was quite unexpected.

The question of the oxygen-sensitivity of E. histolytica is a complex and important one. This organism was long believed to be a strict anaerobe. Cultivation of E. histolytica requires somewhat reduced O_2. On the other hand, neither the tissues invaded by the ameba in vivo nor the culture media used are very O_2-deficient and the amebae have been shown to consume O_2. We approached the problem of oxygen toxicity to E. histolytica by asking how rapidly the cells were killed by near-saturating concentrations of oxygen, and secondly, whether cellular metabolism exerted a protective effect. At 24 and 35 C, the killing curves were biphasic with little loss of viability for the first seven hours, then quite rapid killing. In contrast, at 0 C, there was little killing by oxygen. Thus, amebal sensitivity to O_2 seemed to require metabolic activity. Cells were not protected by superoxide dismutase or catalase.

II Attachment and Motility of Entamoeba:

The cellular processes of attachment, motility and endo- and exocytosis may be important in the invasion and destruction of tissue by E. histolytica. We have continued our studies on the physiological requirements for amebal attachment and motility in the defined "Motility Buffer" (M.B.) we developed. Cysteine and ascorbic acid are essential and probably have a role other than as reducing agents--since they cannot be replaced by N_2. We are now determining the kinetics of attachment and endocytosis in complex growth medium and in M.B. and intend to use it to study interactions between amebae and host cells. Initial experiments indicate that attachment to glass takes place within 3 h in medium or M.B.

Project Description (Continued)

M.B. has been exploited to determine the sensitivity of E. histolytica cells to ultraviolet irradiation since the growth media absorb UV. The reducing agents, cysteine and ascorbic acid protect amebae from U.V. as they do other cells. This may be useful in obtaining amebal mutants.

III Surface Saccharides of Entamoeba:

Evidence has been presented that agglutinability of different Entamoeba by Concanavalin A may be correlated with virulence. All 11 of the "classical" E. histolytica strains (isolated from patients, but varying in virulence in animal models) we tested were strongly agglutinated by Con A. The non-pathogenic E. histolytica-like strains Huff and Laredo were not agglutinated, while some reptilian and free-living Entamoeba were and others weren't. Preliminary experiments indicated that none of these amebae possessed sugars similar or identical to, fucose, or N-acetyl-glucosamine and only one, E. baretti (isolated by Diamond from a turtle) possessed N-acetylgalactosamine-like residues on its external surface. We hope to do similar studies on cysts.

IV Studies with Giardia lamblia:

G. lamblia, is increasingly recognized as a pathogen endemic within the United States. Techniques for the axenic cultivation of the flagellated trophozoite have recently become available and we have initiated studies in several areas.

a) Both attached and "swimming" cells are observed in the host and in culture. Giardia attaches (to glass or intestinal epithelium) with its ventral "sucking disk". We have separated attached from swimming forms and are determining growth and attachment characteristics for each.

b) G. lamblia has a striking requirement for reducing agents for growth. In the absence of cysteine and ascorbic acid, the cells lyse in a few hours. Minus only cysteine, the cells clump, vibrate and don't attach or swim. We are studying the mechanism of this requirement, which we have found to be quite specific.

c) Cell surface: In preliminary studies, we observed that live G. lamblia trophozoites were not agglutinated by Con A, although dead cells were. Thus, it may be possible to enzymatically unmask Con A-binding sites of living trophozoites and thereby analyze their outer surface and that of cysts.

Cysts of G. lamblia from 2 patients were not stained by a fluorescent dye specific for chitin-like polysaccharides. Preliminary studies indicate that G. lamblia cysts may have surface residues similar or identical to N-acetylglucosamine.

Publications:

Gillin, F.D., and Diamond, L.S.: Clonal growth of Entamoeba histolytica and other Entamoeba in agar. Journal of Protozoology (in press).

Gillin, F.D., and Diamond, L.S.: Clonal growth of Entamoeba in agar: some applications of this technique to the study of their cell biology. Arch. Invest. Med. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00161-02 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Immunochemistry of Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI T.E. Nash, Medical Officer, LPD, NIAID

Other: Fouad Boctor, Fogarty Fellow, LPD, NIAID
A.W. Cheever, Assistant Chief, LPD, NIAID
Head, Section on Host Parasite Relations
David Taylor, Fellow, Naval Medical Research Institute
M.A. Kaliner, Medical Officer, LCI, NIAID
W.R. Henderson, Clinical Associate, LCI, NIAID
B. Fraser, Staff Fellow, LIG, NIAID

COOPERATING UNITS (if any) Laboratory of Clinical Investigation, Laboratory of Immunogenetics, NIAID and Naval Medical Research Institute

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host-Parasite Relations Section

INSTITUTE AND LOCATION
NIAID, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
22/12	17/12	5/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Analysis of adult S. mansoni secretory products after labeling in vitro with precursor sugars or amino acids reveals that worms incorporate and then excrete both large and small molecular weight products. The kinetics of excretion, amount of label retained, and the percent released into the media as large molecular weight material is characteristic of the precursor used.

A neutral polysaccharide antigen has been isolated from S. mansoni eggs. This antigen differs from those previously described.

PHS-6040
(Rev. 10-76)

Project Description:

The major effort of the Laboratory continues to be the study of schistosome antigens. Recent studies are directed toward analyzing the kinetics of release and nature of adult secretory materials. Adult worms are cultured in vitro with labeled precursors including N-aceytlglucosamine, N-acetylgalactosamine, fucose, leucine, glucose, sulfate, glucosamine, and galactosamine. At differing time periods worms are then washed and the supernates harvested and separated into large and small molecular weight components. All the precursors studied are taken up by the worms and used to synthesize large molecular weight materials. However, most of the secretory products are small molecular weight. Analysis of the kinetics of release shows that except for fucose and sulfate, both large and small molecular weight secretory products are at first released rapidly and then at a slower linear rate. In contrast, the large and small secretory products of fucose and sulfate are only released rapidly. Additionally, adult worms retain the labeled precursors to different degrees and the percent excreted as large molecular weight material is characteristic of the labeled precursor.

Analysis of the secretory products are in progress. Preliminary experiments show that the number of substances secreted that are labeled with N-acetyglucosamine are relatively large compared to the 1-2 substances found in fucose labeled secretions.

Other experiments are in progress to study factors that control worm secretory processes.

Dr. Fouad Boctor is studying carbohydrate S. mansoni egg antigens. Extraction of S. mansoni eggs with phenol followed by sequential enzymatic treatments results in a fraction essentially free of protein. The fraction contains one identifiable antigen that is stable to proteolytic treatment, boiling, lypholization but is destroyed by periodate. The antigen is neutral at pH 8.2 and has a molecular weight >100,000 daltons; the composition is currently being studied. Elution pattern on DEAE and Con A columns as well as the pattern obtained after acrylamide electrophoresis shows that this antigen differs from the previously described egg antigens. Antibodies to this antigen are present in experimental S. mansoni infections.

Studies involving the interaction of schistosomula with rat mast cells revealed little and sporadic release of superoxide and histamine from mast cells passively sensitized or derived from infected animals.

Studies employing axenically grown Giardia lamblia have begun. Initial experiments concern the development of diagnostic serologic tests.

Publications:

Nash, T.E., Nasir-ud-Din, Jeanloz, R.W.: 1977. Further purification and characterization of a circulating antigen in schistosomiasis. The Journal of Immunology, 119: 1627.

Nash, T.E.: Antibody response to a polysaccharide antigen in schistosomiasis. I. Sensitivity and specificity. Am. J. Trop. Med. Hyg. (in press)

Nash, T.E., Ottesen, E.H., Cheever, A.W.: Antibody response to a polysaccharide antigen present in schistosome gut. II. Modulation of antibody response. Am. J. Trop. Med. and Hyg. (in press).

Amyx, H.L., Asher, D.M., Nash, T.E., Gibbs, C.J., Gagdusek, D.C.: Hepatic amebiasis in spider monkeys. Am. J. Trop. Med. Hyg. (in press)

Weintraub, M., Nash, T.E., Ottesen, E.A., Becker, M.A.: 1977. Uricosuril effects of Niridazole. Clinical Pharmacology and Therapeutics. 22: 568.

Ottesen, E.A., Stanley, A.M., Gelfand, J.A., Gadek, J.E., Frank, M.M., Nash, T.E., Cheever, A.W.: 1977. Immunoglobulin and complement receptors on human eosinophil and their role in cellular adherence to schistosomules. Am. J. Trop. Med. and Hyg. 26: 134.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00162-02 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Biochemical Cytology of Host-Parasite Interactions in Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. M. Dwyer Research Microbiologist, LPD, NIAID

Other: T. B. Fioretti Chemist, LPD, NIAID

COOPERATING UNITS (if any)
Dr. P. A. D'Alesandro, Division of Tropical Medicine, School of Public Health, Columbia University; Dr. K. P. Chang, Laboratory of Parasitology, The Rockefeller University

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Cell Biology and Immunology

INSTITUTE AND LOCATION
NIAID, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
24/12	12/12	12/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Aspects of the cell biology and immunology of host-parasite interactions among several intra- and extracellular parasitic protozoa are being investigated. Leishmania sp. and Trypanosoma sp. are being used as models of intracellular and extracellular parasitism, respectively. Emphasis is placed upon: 1) determining some of the basic chemical and antigenic properties of parasite surface membranes; 2) ascertaining the nature and extent of the interactions of parasite surfaces with specific host cell types; 3) defining the basic mechanisms involved in the intracellular survival and multiplication of parasites within host cells; and 4) attempts to determine the means by which parasites circumvent host immuno-defense systems. Techniques employed in these studies include: subcellular fractionation, ultracyto- and immunochemistry, electron microscopy, affinity chromatography, acrylamide gel gradient electrophoresis, lectin assays, radio isotope labeling, gel immunoassays, and in vitro cell culture.

PHS-6040
(Rev. 10-76)

Project Description and Objectives:

How parasites evade host immune responses, are nourished, multiply, and eventually destroy the host are questions being asked in this investigation. In as much as all initial interactions between host and parasite occur at the level of their respective cell membranes, an understanding of the nature and physiology of these membranes is essential. It is to this end that parasite surface membrane structure, chemical composition, antigenic nature, and physiology are being investigated specifically with regard to leishmaniasis and trypanosomiasis. Further, parasite interactions with specific host cell types and immune systems are being studied to ascertain the role of parasite surface membranes in infectious disease processes.

Methods Employed:

Techniques used include: 1) fine structure (transmission and scanning electron microscopy, ultrastructural cyto- and immunochemistry, freeze fracture and -etching methods, and autoradiography); 2) subcellular fractionation; 3) analytical and preparative polyacrylamide slab gel electrophoresis and isoelectric focusing; 4) antibody and lectin binding assays; 5) radio isotope labeling and autoradiography; 6) chromatography (gel permeation and affinity); 7) qualitative and quantitative gel immunoprecipitin assays; and 8) *in vitro* cell culture.

Major Findings:

1. *Leishmania donovani* - macrophage interactions *in vitro*.

Some parameters of *Leishmania donovani* - hamster bone marrow macrophage interactions were further investigated using an *in vitro* system previously devised in this laboratory. Quantitative studies have indicated that intracellular amastogotes multiply within these cells with a mean generation time of $\sim$ 50 hours. The parasites destroy the host cell *in vitro* between their 8-9th generative cycle which apparently closely resembles the *in vivo* infection. Using a variety of light and electron microscope level markers for lysosomes, quantitative data was obtained which demonstrated that >95% of all intracellular amastigotes reside and multiply within host cell secondary phagolysosomes. Further, fine structure observations suggest the constant fusion of host secondary lysosomes with vacuoles containing the amastigotes. Homologous cytophilic IgG was demonstrated on the surface membrane of *Leishmania* infected bone marrow macrophages using both fluorescein and ferritin labeled antibody techniques. Under permissive conditions the labeled antibody-surface IgG complex as capped, endocytosed, and at least some came to reside within parasite containing vacuoles. Using similar methods, amastigote antigens have been demonstrated to co-exist with host cytophilic IgG on the surface of infected bone marrow cells. However, under permissive conditions such parasite antigens were neither induced to cap nor were they endocytosed by the infected host cell. Fine structure observations also indicate that some amount of parasitophorous vacuolar membrane materials

are recycled by the host cell. The current cumulative results suggest that amastigotes reside and multiply within host cell phagolysosomes, and that a dynamic continuum of membrane exchange and recycling exists between the lysosomal-parasitophorous vacuolar system and the plasma membrane of the infected bone marrow macrophage.

2. Radiolabeling of externally disposed surface membrane constituents of Leishmania donovani promastigotes.

Externally disposed constituents of the Leishmania donovani promastigote surface membrane were radiolabeled using a variety of impermeant, enzyme catalyzed methods. Quantitative results obtained with lactoperoxidase and ^{125}I in various experiments indicated that >96% of all incorporated ^{125}I activity was pellicular membrane associated. The cell surface membrane localization of the ^{125}I label (>95%) was also verified using quantitative fine structure autoradiography. Trypsin, α-chymotrypsin, pronase, and carboxypeptidase-A treatments removed > 49, 72, 76, and 69%, respectively of the total ^{125}I labeled cell activity, and such parasites remained >99% viable. Detergent solubilized labeled cells were separated electrophoretically in sodium dodecylsulfate polyacrylamide continuous gradient slab gels. Autoradiographic and gamma counting results obtained from such gels indicated that a minimum of 23 externally disposed pellicular membrane constituents were specifically labeled with ^{125}I, and these ranged in apparent molecular weight from $<1.45 \times 10^4$ to $>4.0 \times 10^5$ daltons. Many of these components were sensitive to one or more of the above proteolytic enzymes. Some labeled constituents on intact cells were cleaved by carboxypeptidase-A indicating free peptidyl -carboxyl termini, and suggesting their peripheral plasma membrane orientation. Somewhat similar results to those accrued for the ^{125}I labeled cells were also obtained from parasites labeled via the pyridoxal phosphate-NaB^3H_4, and galactose oxidase-NaB^3H_4, and glucose oxidase-NaB^3H_4 oxidation-reduction labeling methods. Results of the latter two methods demonstrated that galactose and, or, N-acetyl-galactosamine, as well as glucose and, or, N-acetyl-glucosamine ligands, respectively, were present as externally oriented constituents in the parasite surface membrane. The current labeling results, with regard to the presence of surface membrane carbohydrates, confirm those previously obtained using various specific lectin binding assays with this parasite. Further, the amount of cell surface associated radioactivity was significantly reduced in galactose oxidase-NaB^3H_4 labelled cells following treatment with α-amylase, dextranase, lysozyme, and β-galactosidase. These results indicate that at least some of the cell surface galactose-like moieties are bonded to oligomeric saccharides which contain repetitive α-1,4- and α-1,6-D-glucan configurations.

A variety of pellicular membrane constituents were commonly labeled by two or more of the various labeling methods employed. The cumulative results of these studies demonstrate that a minimum of 27 peptide and glycopeptide constituents are present on the external surface of L.

donovani pellicular membranes, and presumably some of these are major parasite cell surface antigens.

3. Isolation and characterization of parasite cell surface membranes.

Improved centrifugation methods were devised for the isolation of highly purified preparations of Leishmania donovani promastigote pellicular membranes (PM). The isolated PM had an apparent density equivalent of $\sim$ 1.24 g/cm^3. As ascertained by electron microscopy, subpellicular microtubules (MT) remained structurally attached to the inner surface of the isolated PM, and this characteristic was used to assess membrane fractions for purity. Gradient fractions having >95% of all included membranes associated with MT were obtained routinely. Typically, $\sim$ 4 mg of total PM protein was obtained from 5 x 10^{10} promastigotes. Membrane asymmetry with regard to the external surface lamina orientation of glycoslated ligands was demonstrated in PM using a concanavalin A-ferritin conjugate. Cationized ferritin binding, indicative of negative surface change, occurred on both the inner and outer PM lamina, and in the latter instance caused displacement and aggregation of the associated MT. Dissolution of the PM bilayer occurred after Triton X-100 extraction; however, the spacial configuration of the associated MT nexus residuum remained structurally intact. These results suggest that MT are bound both to each other and to the surface membrane via a filamentoid network. Sodium dodecyl sulfate-polyacrylamide slab gel electropherograms (SDS-PAGE) of isolated PM characteristically contained $\sim$ 40 peptide bands ranging in molecular weight (MW) from <1.2 x 10^4 to 2.8 x 10^5 daltons. Results of the SDS-PAGE peptide MW analyses of isolated PM are highly correlative with those results independently obtained in the various surface membrane radiolabeling studies outlined in section #2 above. The major peptide species present in both SDS-PAGE of the intact and Triton insoluble residue of PM had a MW of $\sim$ 5.3 x 10^4 daltons, and was presumed as the constituent MT protein, tubulin. This band was absent in SDS-PAGE or the PM Triton extract. In gel diffusion reactions rabbit anti-promastigote and -amastigote sera gave 2 precipitin lines of antigenic identity with the PM Triton extract indicating, that both parasite developmental stages share at least 2 common or cross-reacting surface membrane antigens. Further, one of the latter membrane antigens was also immunologically detected as an "exo-antigen" in sera obtained from L. donovani infected hamsters.

Preparative isolation of various isolated PM constituents is currently being pursued for future studies concerning their chemical and immuno-chemical composition. Further, antibodies are currently being raised in goats and rabbits against individual PM components, and these should prove useful as probes for dissecting and characterizing the surface membrane of the corresponding intracellular amastigote stage of the parasite.

Proposed Course:

1. Various immunochemical and lectin probes will be used to further elucidate the nature of the intact parasite surface membrane.

2. Chemical, immunochemical, and lectin binding ligands will also be used in the preparative isolation of specific parasite membrane constituents for further detailed chemical and immunochemical analyses (e.g. total carbohydrate and amino acid analyses of isolated membrane constituents in collaboration with individuals in the Laboratory of Immunogenetics/NIAID).

3. The total composition and characterization of parasite membrane lipids will be investigated (i.e. in collaboration with Dr. Edger Ribi's group at the Rocky Mountain Laboratory, NIAID).

4. Studies will be pursued concerning the nature and cellular origin of parasite exoantigens present in host circulation, and their possible effects on the state of host immune responsiveness.

5. Interaction and fate of individual isolated parasite membrane constituents will be studied with specific host macrophage populations.

Significance:

Basic research concerning the molecular and structural composition of specific protozoan parasite surface membranes and their interactions with specific host cell types might provide a basis for future immunoprophylaxis programs and regimens and, or, a more rational approach toward the development of effective delivery systems for chemotherapeutic treatment of these diseases of man.

Publications:

1. Chang, K.-P. and Dwyer, D.M.: *Leishmania donovani* hamster macrophage interactions *in vitro*: cell entry, intracellular survival, and multiplication of amastigotes. *J. Exp. Med.* 147: 515-530, 1978.

2. Dwyer, D. M.: Membrane interactions between *Leishmania* and host cells. *In* Schlessinger, D. (Ed.): *Microbiology-1977*. Washington, D. C., American Society of Microbiology, In press.

3. Dwyer, D. M.: The surface antigens of parasitic protozoa. *In* Hutner, S. (Ed.): *Protozoological Actualities-1977*. Lawrence, Kansas, Allen Press, In press.

4. Dwyer, D. M.: The recent advances in *in vitro* cultivation methods of trypanosomatids. In Hutner, S. (Ed.): *Protozoological Actualities-1977*. Lawrence, Kansas, Allen Press, In press.

5. Dwyer, D. M. and D'Alesandro, P. A.: Trypanosoma lewisi: subcellular fractionation and immunochemical identification of "exoantigens" as surface membrane constituents. Exp. Parasitol., In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00174-01 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Culture, physiology and antigenic analysis of sexual and asexual erythrocytic malaria parasites.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Carter	Visiting Scientist
	L.H. Miller	Head, Malaria Section
Other:	R. Gwadz	Senior Scientist
	J. Johnson	Visiting Associate
	R.F. Beach	Staff Fellow
	S.K. Martin	Visiting Associate
	F.M. McAuliffe	Staff Fellow
	T. Shiroishi	Biologist

COOPERATING UNITS (if any)
M. Aikawa, Case Western Reserve University, Cleveland, Ohio; J.D. Haynes, Dept. of Immunology, WRAIR; J. Kark, Dept. of Hematology, WRAIR.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
116/12	79/12	37/12

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Animals have been successfully vaccinated against sexual and asexual malaria parasites. In addition, Plasmodium falciparum, the major malaria of man, has been grown in culture for the production of asexual stages and gametocytes. The present study will 1) Improve culture conditions for the production of large numbers of gametocytes, gametes and merozoites of P. falciparum; 2) Study of the physiology of exflagellation, fertilization and invasion of red cells by malaria merozoites; 3) Characterize structure, function and immunogenicity of surface determinants on gametes and merozoites; 4) Evaluate gamete vaccines in model systems for identification of the best antigens and adjuvants.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. Improvement of culture conditions for sexual and asexual parasites of Plasmodium falciparum. This will include methods of mass cultivation, methods for fractionation of asexual and sexual parasites, and attempts to understand the controls on gametocytogenesis and development of gametocytes in culture.

2. Study of the physiology of gamete formation, fertilization and merozoite invasion of RBCs.

3. Structure, function and immunogenicity of antigenic determinants on sexual and asexual parasites.

4. Study of immunogenicity of crude antigenic preparations of sexual and asexual stages in model systems.

Each objective is interrelated with the others: Culture will be needed for production of antigenic material for analysis of both sexual and asexual erythrocytic parasites. Structure and function of parasite determinants will be studied in the framework of parasite physiology. Successful immunization with whole parasite preparations will identify sources of more purified immunogenic materials.

Methods:

1. Continuous culture of red cell stages of Plasmodium falciparum; isolation of sexual and asexual stages; purification of merozoites and gametes.

2. Use of model systems: P. gallinaceum-chicken for study of gametes and gamete immunity; P. knowlesi-rhesus for study of mechanism of invasion and gamete immunity; P. berghei-rodent for study of gamete immunity.

3. Use of polyspecific antibody to parasite antigens for immune precipitation; high-resolution gel electrophoresis and electrofocussing separation techniques; ^{125}I lactoperoxidase surface labelling; general metabolic labelling of all parasite components.

4. Monospecific antibody produced in hybridomas for analysis of parasite surfaces and isolation of surface determinants.

Major Findings:

1. Following the growth of functionally mature P. falciparum gametocytes in culture (Carter and Beach, 1977), efforts have been devoted to increasing their yield and viability. Some success has been achieved using medium conditioned with diploid human fibroblast cell cultures and an occasional

mature gametocyte that exflagellates has been observed. Isolates vary in the production of gametocytes.

2. A method has been developed for preparing extracellular macrogametes, unexflagellated microgametocytes and microgametes of P. gallinaceum.

3. A method for the production of purified viable, invasive merozoites of P. knowlesi in a serum-free medium has been developed.

4. Following the observation that children with severe falciparum malaria have a higher RBC pyridoxal kinase than the general population, we have studied the requirement of host pyridoxal kinase for parasite growth. Three parasites (P. falciparum, P. gallinaceum and P. knowlesi) have no detectable pyridoxal kinase. All host RBCs (with the possible exception of turkeys) have pyridoxal kinase; cats, dogs, cows and horses have none.

5. The studies on the Duffy-vivax hypothesis have been completed with the study of Vietnam veterans who were black and infected with vivax malaria. All twelve veterans studied had the Duffy positive phenotype; the frequency of the Duffy positive phenotype in the black U. S. population is approximately 50%.

6. We have developed a method for isolation of the attachment phase of RBC invasion. Cytochalasin-treated merozoites attach specifically to RBCs from susceptible hosts (rhesus monkeys and man) and form a junction between the apical end of the merozoite and the RBC. Cytochalasin-treated merozoites will also attach specifically to Duffy blood group negative human RBCs, although these human RBCs are refractory to invasion. The mechanism of attachment, however, differs in that there is no junction formation with Duffy negative RBCs.

7. Studies have demonstrated that preparations containing male and female gametes of P. gallinaceum immunize chickens in such a way as to prevent infectivity to mosquitoes during subsequent induced blood infections. Purified male or female gametes are less immunogenic than the crude mixed preparation.

8. Monkeys immunized up to 2 years ago with a single inoculation of a P. knowlesi antigen preparation containing 10^6 to 10^7 microgametes with lesser numbers of macrogametes and asexual trophozoites in Freund's complete adjuvant (FCA) continue to show complete transmission blocking immunity. These monkeys do not infect mosquitoes when challenged with with homologous or heterologous strains of P. knowlesi. This immunity persists even in splenectomized monkeys. Protection against asexual stages persists in intact animals, but after splenectomy, immunized monkeys cannot control their infections.

Various adjuvants have been evaluated as alternatives to FCA. The antigen preparation plus Freund's incomplete adjuvant induced complete although transient antigamete immunity in 3 of 4 monkeys. Neither BCG

or DPT was effective.

9. Sera from adult immune Gambians did not block oocyst formation by gametes of P. falciparum. The sera did not react with surface antigens on P. falciparum gametes by fluorescent antibody (FA) techniques. In addition, sera from monkeys immunized with P. knowlesi sporozoites or merozoites did not react with gametes.

Proposed Course:

1. Culture.

   a. Evaluate promoters, conditioned medium and PABA reduction in medium on gametocyte production.

   b. Develop steady state culture conditions (e.g., fiber chamber, spinner) for growth to high parasitemia.

   c. Replacement or reduction in requirement for human serum.

   d. Development of in vitro cloning methods for evaluation of gametocytogenesis.

   e. Develop techniques for separation of gametocytes and asexual parasites.

2. Determine the chemical nature of mosquito exflagellation factor (MEF) by column fractionation and mass spectroscopy.

3. Analyze surface antigens on gametes and merozoites. Attempts will be made to study the function of these structures (e.g., attachment to receptors) and immunogenicity.

4. Development of assay to evaluate candidate compounds as receptors for merozoite-RBC attachment. Liposomes with incorporated RBC membrane proteins will be used as receptors of merozoites or blockers of invasion.

5. Study RBC membrane components of the invaginated RBC membrane containing the merozoite, the parasitophorous vacuole.

6. Development of hybridomas to make monospecific antibody against antigen on P. gallinaceum gametes, P. knowlesi merozoites and asexual and sexual stages of P. falciparum. These will be used as probes for the study of surface determinants on parasites and on affinity columns for the isolation of antigens.

7. Evaluation of immunogenicity of purified male or female gametes, mixed gametes and unfractionated parasites in the induction of immunity against infectivity of mosquitoes by P. gallinaceum.

8. Evaluation of adjuvants for induction of immunity in the P. knowlesi-gamete system. Development of the P. berghei-rodent system for the evaluation of adjuvants against mosquito infectivity by gametocytes.

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Carter, R. and Walliker, D. Biochemical markers for strain differentiation in malarial parasites. Bull. Wld. Hlth. Org., 55: 339-345, 1977.

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Nijhout, M. M. and Carter, R. Gamete Development in Malaria Parasites. I. In vitro stimulation by pH and Bicarbonate. Parasitology, 76: 39-53, 1978.

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Martin, S. K., Miller, L. H., Nijhout, M. M. and Carter, R. Plasmodium gallinaceum: Induction of Male Gametocyte Exflagellation by Phospho-diesterase Inhibitors, 44: 239-242, 1978. (Exp. Parasitol.)

Martin, S. K., Miller, L. H., Kark, J. A., Hicks, C. U., Hant, N. J., Okoye, V. C., Esan, G. J. F., Low Erythrocyte Pyridoxal-kinase Activity In Blacks: it's Possible Relation to Falciparum Malaria, Lancet, 1: 466-468, 1978.

Nijhont, H. F., Martin, S. K., Alpha Adrenergic Activity of Isoproterenol In Mosquito Antennae. Experientia, In press

Martin, S. K., Miller, L. H., Hicks, C. U., David West, A., Ugbode, C., Deane, M., Frequency of Blood Group Antigens In Nigerian Children with Falciparum Malaria, Trans. Roy. Soc. Trop. Med. & Hyg. In press.

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Gwadz, R. W. and Green, I. Malaria immunization in rhesus monkeys: A vaccine effective against both the sexual and asexual stages of Plasmodium knowlesi. J. Exp. Med. (In press)

Miller, L. H. Hypothesis on the mechanism of invasion of erythrocytes by malaria merozoites. Bull WHO. 55: 157-162, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00105-05 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Mechanism of Invasion of Erythrocytes by Malaria Merozoites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L.H. Miller	Head, Malaria Section, LPD,NIAID
Others:	F. McAuliffe	Staff Fellow, LPD,NIAID
	J. Johnson	Visiting Associate, LPD,NIAID
	J.A. Dvorak	Research Microbiologist, LPD,NIAID
	T. Shiroishi	Zoologist, LPD,NIAID
	S.K. Martin	Visiting Associate, LPD,NIAID
	M. McGinniss	Research Biologist, BB, CC
	R. Asofsky	Chief, Laboratory of Microbial Immunity, LMI,NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To give a better focus to the overall research of the Malaria Section this project has been combined in Project No. Z01 AI 00174-01 LPD.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00110-05 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Gamete-Specific Immunity in Malaria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.W. Gwadz	Research Entomologist, LPD,NIAID
Others:	R. Carter	Visiting Scientist, LPD,NIAID
	David Chen	Staff Fellow, LPD,NIAID
	Ira Green	Medical Director (Research), LI,NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To give a better focus to the overall research of the Malaria Section this project has been combined in Project No. Z01 AI 00174-01 LPD.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00150-02 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Enzyme Variation in Malaria and Babesia Parasites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Carter	Visiting Scientist, LPD,NIAID
Others:	L.H. Miller	Head, Malaria Section, LPD,NIAID
	T. Shiroishi	Zoologist, LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To give a better focus to the overall research of the Malaria Section this project has been combined in Project No. Z01 AI 00174-01 LPD.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00159-01 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Physiology and Culture of Sexual Stages of Malaria Parasites.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Carter	Visiting Scientist
Others:	D.H. Chen	Staff Fellow
	S.K. Martin	Visiting Fellow
	R.F. Beach	Staff Fellow
	L.H. Miller	Head, Malaria Section
	F.M. McAuliffe	Staff Fellow
	M.M. Nijhout	Staff Fellow
	A.I.O. Williams	Guest Worker

All of the above LPD,NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To give a better focus to the overall research of the Malaria Section this project has been combined in Project No. Z01 AI 00174-01 LPD.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00113-06 LPD

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on dengue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Leon Rosen, Head, Pacific Research Section, LPD, NIAID

Others: Robert B. Tesh, Staff Member, Pacific Research Section, LPD, NIAID
Jih Ching Lien, US Naval Medical Research Unit No. 2, Taipei, Taiwan
John H. Cross, US Naval Medical Research Unit No. 2, Taipei, Taiwan

COOPERATING UNITS (if any)

US Naval Medical Research Unit No. 2, Taipei, Taiwan

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Pacific Research Section

INSTITUTE AND LOCATION

NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Transovarial transmission of all four serotypes of dengue virus was demonstrated in Aedes albopictus but not Aedes aegypti mosquitoes.

Dengue viruses were shown to replicate well in most species of Aedes mosquitoes tested but not in mosquitoes of other genera, with the exception of Toxorhynchites.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives

1. To study the epidemiology and pathogenesis of dengue and dengue hemorrhagic fever.

2. Specifically, to assess a), the risk of primary versus secondary dengue infections with respect to the development of dengue shock syndrome and other serious clinical manifestations of infection and b), the relative pathogenicity of the four different dengue serotypes.

Methods Employed

Aedes albopictus, Aedes aegypti, and other species of mosquitoes were infected with various strains of dengue virus by intrathoracic inoculation or by feeding on virus-containing mixtures. For transovarial transmission experiments the progeny of these mosquitoes were killed at various stages of their life cycle and assayed for virus content by the intrathoracic inoculation of Toxorhynchites mosquitoes. Mosquitoes were assayed quantitatively for virus content by inoculating serial dilutions of triturated insects into groups of Toxorhynchites mosquitoes. Toxorhynchites were examined for virus infection by direct fluorescent microscopy.

Major Findings

Although a large amount of work remains to be done on material previously collected in Rangoon, Burma, most of the effort during the year was devoted to studies on transovarial transmission of dengue viruses.

Transovarial transmission of dengue types 1, 2, 3, and 4 was demonstrated in a Hawaiian strain of Aedes albopictus but not in a Taiwan strain of Aedes albopictus nor a Taiwan strain of Aedes aegypti. There was a considerable variation in the percentage of the F_1 generation infected when different serotypes and different strains of the same serotype were compared. There did not appear to be a correlation between the extent of virus replication in the mosquito and the likelihood of transovarial transmission. Transovarial transmission was demonstrated with a high mouse passage strain of dengue type 1 as well as with numerous strains of dengue viruses which had not been passed in mice or cell cultures.

Transovarial transmission of dengue virus also was demonstrated when parent female mosquitoes were infected by the oral route.

Dengue viruses were shown to replicate well in six species of the Aedes subgenus Stegomyia (aegypti, albopictus, desmotes, albolineatus, malikuli, and alcasidi) and in Toxorhynchites aurifluus; fairly well in Tripteroides bambusa, Heizmannia taiwanensis, and Aedes togoi; poorly in Armigeres subalbatus and

Topomyia yanbarensis; and not, or minimally, in Culex species (pipiens, vishnui, tritaeniorhynchus) and Anopheles species (sinensis and stephensi). They also did not replicate in Drosophila melanogaster.

Significance to Biomedical Research and the Program of the Institute

On a world-wide basis, the four dengue serotypes collectively are by far the most important viruses transmitted to man by arthropods, whether measured by the number of infections or the number of deaths. Millions of dengue infections occur annually and dengue hemorrhagic fever is a leading cause of hospitalization and death among children in heavily populated Southeast Asia. Although more prevalent in Southeast Asia, dengue infections occur or have occurred in most parts of the world warm enough for survival of the mosquito vectors. Unlike most other arthropod-borne viral infections of man, the usual source of infection for transmitting mosquitoes is not a wild or domestic lower vertebrate, but rather, man himself. Because of this unusual epidemiologic feature and the adaptation of the mosquito vectors to urban environments, the prevalence of dengue infection has increased enormously in recent times - the result of increasing urbanization of human populations throughout the world.

Despite the importance of disease in man produced by dengue viruses its pathogenesis is very poorly understood. For example, it is not known in which cells or tissues the virus replicates in vivo. In large part, this lack of information is the result of difficulties of working with this group of viruses in the laboratory. Unlike most other arthropod-borne viruses which affect man, dengue viruses are not very pathogenic for laboratory mice, nor do they commonly produce cytopathic effects in cell cultures. Moreover, primates are the only vertebrates which can be infected with dengue viruses by a natural route (i.e. mosquito bite) and no primate, other than man, exhibits signs of disease following infection.

The demonstration of transovarial transmission of dengue viruses in the laboratory adds a new dimension to the epidemiology of the agents and is significant for control procedures. It is important to determine what role such transmission plays in the maintenance of dengue viruses in nature.

Proposed Course of the Project

The Pacific Research Section will cease to be a part of the NIAID intramural program on October 1, 1978. Work on the current project will be continued under contract with the Research Corporation of the University of Hawaii.

Publications

Gubler, D. J., Reed, D., Rosen, L., and Hitchcock, J. C., Jr.: Epidemiologic, clinical, and virologic observations on dengue in the Kingdom of Tonga. Am. J. Trop. Med. Hyg. 27:581-589, 1978.

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Rosen, L.: Hemorrhagic fevers. In Rothschild, H. (ed.): Human Diseases Caused by Viruses. New York, N. Y., Oxford University Press, 1978, pp. 133-145.

Rosen, L.: Reoviruses. In Lennette, E. H. and Schmidt, N. J. (eds.): Diagnostic Procedures for Viral and Rickettsial Infections, (ed. 5). New York, N. Y., American Public Health Association. (in press).

Rosen, L.: Arthropods as hosts and vectors - experimental infections. In Schlesinger, W. (ed): Togaviruses. New York, N. Y., Academic Press, Inc. (in press).

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NOTE: Please see Project Number Z01 AI 00164-02 LPD for technical support provided.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI 00115-04 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies on the natural history of influenza virus in swine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Gordon D. Wallace, Staff Member, Pacific Research Section, LPD, NIAID

COOPERATING UNITS (if any)
Animal Science Department, College of Tropical Agriculture, University of Hawaii and Division of Animal Industry, Hawaii State Department of Agriculture, Honolulu, Hawaii

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Pacific Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Data from prospective seroepidemiologic studies continue to support the hypothesis that swine influenza virus (Hsw1N1) is maintained in swine herds by an unusual method, not involving lungworms, perhaps by chronic or latent infections in individual swine. However, it appears that current human subtypes of influenza A virus (H3N2 and H1N1) have not become established in swine in Hawaii. Limited experimentation with swine influenza virus on transplacental transmission and neonatal infection in swine, including the detection of virus by organ culture, has failed to provide insight into possible biologic mechanisms that allow the virus to survive in apparently immune herds.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives

To determine: 1) if latent or chronic influenza virus infection in swine is a method by which the virus is maintained in swine herds during nonepidemic periods, 2) what factors are critical to epidemic spread of influenza virus in swine, 3) the frequency and epidemiologic importance of interchange of influenza viruses between swine and man.

Methods Employed

Observations on the epidemiology of influenza in swine indicate that swine influenza virus (HswlN1) is maintained in swine herds by methods other than periodic reintroduction from the outside. Dr. Richard Shope developed the hypothesis, supported by extensive experimental data, that swine lungworms residing in their earthworm intermediate hosts can harbor a masked form of swine influenza virus, which becomes infectious after the lungworm is acquired by a pig. Other experimental and epidemiologic data, however, suggest that swine not infected with lungworms may develop latent influenza infections with subsequent reshedding of virus. Retrospective and prospective seroepidemiologic and virologic studies, initially designed to evaluate the role of lungworms and influenza virus latency in the epidemiology of swine influenza, have been in progress in Hawaii since 1974. (Advantages of conducting such studies here include the relative isolation of the islands and the apparent regular occurrence of swine influenza in swine herds not infected with lungworms.) Carefully selected study-herds have been periodically tested for hemagglutination inhibiting (HI) and neutralizing antibodies to A/swine (HswlN1) and current type A human influenza viruses (H3N2 and H1N1). A large number of blood samples collected from other herds for brucellosis tests have also been tested for influenza antibodies. In addition to the field work, pregnant sows and newborn pigs were experimentally exposed to swine influenza virus to determine if transplacental transmission and latent or chronic infections might result.

Major Findings

Observations in nature. As reported last year, an epidemic of swine influenza was encountered on the island of Oahu in November-December, 1976. Epidemiologic data suggested that swine influenza virus had been maintained in individual herds by latent or chronic infection in breeding animals, with epidemics occurring when herd immunity reached low levels (about every 3 years). Apparently lungworms were not involved. Continued serologic surveillance on Oahu has further supported the hypothesis that virus is continuously maintained in certain herds. For example, at intervals of one or 2 months since the epidemic, some of the feeder pigs (5-8 months of age) sent to slaughter from about 30 herds involved in the epidemic have been sampled. Of a total of 1,254 pigs from these herds tested since August, 1977, 37% had HI antibody ($\geq$1:20) to swine influenza virus, whereas pigs from most of the herds not in the epidemic remained negative, including pigs in a large number (72) of herds tested

on 3 other Hawaiian islands. Furthermore, after it was established that most if not all swine in one closed herd had been infected with swine influenza virus during the epidemic period (early December, 1976); continued surveillance revealed that 20-90% of pigs 4-6 months old, born up to 14 months later (last period sampled), had specific HI antibody, many at high titer (≥1:80) to swine influenza virus. Very few if any would have had maternal antibody at the age tested, since the half life of passively transmitted IgG in swine is 9 days. (Antibody titers in sera of the pigs tested were not reduced by treatment with 2-mercaptoethanol, with 2 exceptions). No swine had been introduced into this herd since the epidemic, and it had been free of lungworms.

All swine sera collected also have been tested for antibody to the A/Hong Kong human subtype (H3N2) of influenza virus and 3 of its variants: A/England, A/Port Chalmers, and A/Victoria. In addition, sera collected since April, 1978, were also tested for antibody to the A/Russian subtype (H1N1). Whereas about 20% of herds tested in 1976 were found to have swine with H3N2 antibody (A/Eng or A/PC), only 2 herds were found infected in 1977 (A/Victoria), and only one animal has been discovered with a high titer to A/Russian virus.

Experimental results. Of 19 pigs born to 3 sows experimentally infected with swine influenza virus 10, 24, and 39 days respectively before parturition, 3 pigs from each litter have been killed for examination. Two from each litter were deprived of colostrum and killed (one born dead) within 24 hours of birth; the others at later dates. Isolation of virus was attempted directly from lung tissue and also from culture media of explants of lungs, trachea, or bronchi, of all pigs, and from various other organs, and explants of organs, from some of the pigs. These organs included liver, spleen, bladder and kidney. Isolation of virus from placentas and amniotic tissues on newborns was also attempted. Virus was detected in only one pig. This isolation was made (and confirmed by re-isolation) from culture medium of a trachea explant from a pig whose mother had been infected 10 days before parturition. Although all of the evidence indicates that the pig was infected in utero (transplacentally), transmission of virus from the sow to her pig right after birth cannot be completely ruled out.

Eleven other pigs from uninfected mothers were inoculated intranasally with virus within 2 hours of birth (before nursing). All but one either died or were killed from 8 to 70 days after exposure. Throat swabs were collected from all pigs daily (except on weekends) for 4 weeks, or until death, and urine was collected periodically from a few. Lung tissue from all pigs was tested directly for virus by the inoculation of tissue suspensions into amniotic and allantoic cavities of developing chick embryos. In addition, explants of lungs and trachea or bronchi were established and culture media were collected weekly for 3 weeks and tested likewise for virus. From 4 of the pigs, other organs and tissues including spleen, liver, kidney, bladder, and heart (also urine from 2) were tested for virus directly. Explants of some of these organs were established and media tested.

Gross pathology in lungs, sometimes extensive, was observed in most of the pigs. Virus was isolated from throat swabs of all the pigs - as long as 10 and

11 days after exposure from 3. Virus was isolated from lungs of 2, killed on days 8 and 14. No other virus isolations have been made to date, however some results are pending.

Earlier, a group of 5 5-day-old pigs had been exposed and tested similarly to those described above. They were killed between days 20 and 67. One was given daily injections of cortisone for 14 days prior to examination. In addition to organs and urine, blood was collected daily for 5 days to test for viremia. There was no evidence of disease in any of these pigs. Virus was isolated from throat swabs of all up to 7 days after exposure and from one at 10 days. Virus was also isolated from the blood of one pig on days 2 and 5.

Significance to Biomedical Research and the Program of the Institute

New information on the natural history of swine influenza virus is of considerable biologic interest and might provide insight into the natural history of human influenza.

Proposed Course of the Project

Terminated August 1, 1978, Dr. Wallace is transferring to Bethesda, Maryland.

Publications

None

NOTE: Please see Project Number Z01 AI 00164-02 LPD for technical support provided.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00163-02 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Field and laboratory studies on the transovarial transmission of Japanese and St. Louis encephalitis viruses by mosquitoes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Leon Rosen, Head, Pacific Research Section, LPD, NIAID

Others: Robert B. Tesh, Staff Member, Pacific Research Section, LPD, NIAID
Jih Ching Lien, US Naval Medical Research Unit, No. 2, Taipei, Taiwan
John H. Cross, US Naval Medical Research Unit, No. 2, Taipei, Taiwan
James Hardy, School of Public Health, University of California, Berkeley, California
Kennedy F. Shortridge, Department of Microbiology, University of Hong Kong, Hong Kong, B.C.C.
Han Kee Hong, Department of Virology, National Institute of Health, Seoul, Korea

COOPERATING UNITS (if any) NAMRU-2, Taiwan; University of California, School of Public Health; University of Hong Kong, Department of Microbiology; National Institute of Health, Department of Virology, Seoul, Korea.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Pacific Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.7	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Various species of female mosquitoes were infected with Japanese or St. Louis encephalitis viruses either by intrathoracic inoculation or by feeding on virus-containing mixtures. The F_1 progeny of these mosquitoes were tested for the presence of virus by inoculation of Toxorhynchites mosquitoes. Transovarial transmission of Japanese encephalitis virus was demonstrated in Aedes albopictus, Aedes togoi, Aedes aegypti, Armigeres subalbatus, and Culex pipiens pallens. Transovarial transmission of St. Louis encephalitis virus was not demonstrated in the experiments carried out thus far.

Larvae and pupae collected in the field in Japanese encephalitis endemic areas in Taiwan, Hong Kong, and Korea are being tested for the presence of Japanese encephalitis virus by the inoculation of Toxorhynchites mosquitoes.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives

To determine if transovarial transmission of Japanese and St. Louis encephalitis viruses by mosquitoes plays a role in the survival of these viruses in nature when environmental conditions are unfavorable for their vectors.

Methods Employed

Various species of female mosquitoes (from laboratory colonies or field-caught) were infected with Japanese or St. Louis encephalitis viruses either by intrathoracic inoculation or by feeding on virus-containing mixtures. The F_1 progeny of these mosquitoes were killed at various stages of development and assayed for virus content either by the intrathoracic inoculation of Toxorhynchites mosquitoes or the inoculation of Vero cell cultures. Toxorhynchites were examined for virus infection by direct fluorescent microscopy. F_1 generation mosquitoes also were tested for their ability to transmit virus to vertebrate hosts.

Larvae and pupae collected in the field in Japanese encephalitis endemic areas in Taiwan, Hong Kong, and Korea were tested for Japanese encephalitis virus by inoculating triturated suspensions of these aquatic stages, or imagoes reared from them in the laboratory, into T. amboinensis mosquitoes. The latter were examined for virus infection as described above.

Major Findings

Transovarial transmission of Japanese encephalitis (JE) virus was demonstrated in the laboratory in Aedes albopictus (Hawaiian and Taiwan strains), Aedes togoi (Taiwan and Japanese strains), Aedes aegypti, Armigeres subalbatus, and Culex pipiens pallens. The percentage of F_1 generation mosquitoes infected ranged from approximately 1 in 80 (A. subalbatus) to less than 1 in 5,000 (C. pipiens pallens). It also was demonstrated that transovarially infected mosquito larvae developed at a slower rate than did uninfected larvae. Transovarial transmission of JE virus was not found with Culex tritaeniorhynchus or Culex vishnui, classical vectors of JE to man in Asia.

Female A. albopictus infected with JE by transovarial transmission infected newly hatched chickens by feeding on them.

Transovarial transmission of St. Louis encephalitis virus was not found in A. albopictus, Aedes dorsalis, or Aedes triseriatus. Both a high and low mouse passage strain of virus was used in studies with A. albopictus but only a low mouse passage strain was employed in work with the latter two mosquito species.

Extensive collections of mosquito larvae and pupae have been made in JE endemic areas in Taiwan and Hong Kong and it is anticipated that similar collec-

tions will have been made in Korea before the end of the period covered by this report. Laboratory tests of this material for the presence of JE virus have just begun.

In the Hong Kong studies it was demonstrated that JE virus was endemic on Hong Kong Island despite the absence of the classical vectors of JE, namely C. tritaeniorhynchus and C. vishnui.

JE virus failed to replicate when inoculated into Drosophila melanogaster but did so following inoculation in each of 17 species of mosquitoes (genera Aedes, Culex, Anopheles, Armigeres, Tripteroides, Topomyia, Heizmannia, and Toxorhynchites).

Significance to Biomedical Research and the Program of the Institute

The mechanism by which mosquito-borne viruses survive periods of adverse environmental conditions for their insect hosts still is largely unknown. Various hypotheses have been proposed including chronic or recurrent viremia in vertebrate hosts, periodic re-introduction of virus by migrating birds or bats from more favorable environments, and persistence of virus in species of mosquitoes or other arthropods which survive adverse conditions in the adult stage. Although survival by vertical transmission in mosquitoes was one of the earliest hypotheses considered, it was largely abandoned when extensive and careful laboratory and field studies failed to confirm initial positive reports. If it can be demonstrated that JE and St. Louis encephalitis virus do survive adverse environmental conditions by transovarial transmission, then more effective control (or even eradication from certain geographic areas) may be possible.

Proposed Course of the Project

The Pacific Research Section will cease to be part of the NIAID intramural program on October 1, 1978. Work on the current project will be continued under contract with the Research Corporation of the University of Hawaii.

Publications

Rosen, L., Tesh, R. B., Lien, J. C., and Cross, J. H.: Transovarial transmission of Japanese encephalitis virus by mosquitoes. Science 199:901-911, 1978.

NOTE: Please see Project Number Z01 AI 00164-02 LPD for technical support provided.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00164-02 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Epidemiology of Infectious Disease in the Pacific Region

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Dean	Pacific Center for Geographic Disease Research - Honolulu under Contract to LPD, NIAID (Contract No. N01-AI-02228)
Other:	L. Rosen	Head, Pacific Research Section and Project Officer, Contract N01-AI-02228 LPD, NIAID
	R. Tesh	Staff Member, Pacific Research Section LPD, NIAID
	G. Wallace	Staff Member, Pacific Research Section LPD, NIAID

COOPERATING UNITS (if any)
Department of Pediatrics, University of Hawaii School of Medicine, Honolulu; Department of Medical Research, Rangoon, Burma.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Pacific Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
24/12	12/12	12/12

CHECK APPROPRIATE BOX(ES)

XX (a) HUMAN SUBJECTS XX (b) HUMAN TISSUES ☐ (c) NEITHER

XX (a1) MINORS XX (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This contract (NO 1-AI-02228) extends professional research capability (Dr. Dean) of the field lab of the Pacific Research Section and provides technical support for the existing professional staff of the Section.

Dr. Dean's research program is designed to elucidate the epidemiology and public health significance of newly discovered viral and other infectious agents of diarrheal disease in man. Neonates in two Honolulu hospitals were found to have a zero prevalence of rotavirus infection. This contrasts with studies in Britain and Australia where the prevalence was 33% and 49%. These differences have important implications for immunity to rotavirus in later childhood.

An epidemiologic and microbiologic study of the largest epidemic of Kawasaki Disease ever reported in the U.S. is underway.

Technical support under the contract has been mainly for the dengue and arbovirus vector research. (See Projects Z01-AI-00113-06, 00163-02, 00115-04, 00177-01 and 00178-01).

PHS-6040
(Rev. 10-76)

Project Description:

Objectives

The objective of this project is to elucidate the etiology and epidemiology of selected infectious diseases in the Pacific region. Primary emphasis is on (1) infections whose epidemiology may be studied more effectively in isolated island populations of the Pacific and (2) on vector-transmitted infections.

Objectives of the project are implemented in two ways:

(1) Support of a professional capable of conducting his own independent research program, and

(2) Providing technical support for existing professional field staff of the Section and their research programs.

Methods Employed

Conventional methods for isolation of infectious agents such as bacterial culture, cell culture and animal inoculation are used, as well as immuno-electron microscopy. Serologic tests that are used for demonstration of antibodies include virus neutralization, hemagglutination-inhibition, fluorescent antibody, gel diffusion, etc. In addition, an insectary is maintained to provide mosquitoes for tests of virus transmission and for recently developed techniques of direct intrathoracic inoculation of mosquitoes for isolation of certain arboviruses.

Conventional epidemiological methods are used. Serum samples on population groups are stored frozen for serologic surveys.

Major Findings

Since April 1977, 19 patients and 8 families containing 23 other individuals have been investigated in the Honolulu Study. The agents so far identified in the 19 patients' stools are:

Rotavirus	5
Salmonella	2
E. coli producing LT	0
E. coli producing ST	0
Yersinia	0
Campylobacter	0
Vibrio parahemolyticus	0
Mycoplasma - By electron microscopy	2
Candida	2

So far no rotaviruses or enterotoxigenic E. coli have been found in the stools of family members.

Serologic studies of rotavirus antibody by complement fixation give results as follows in sera from Pacific Islands:

	Sera Tested	% Positive (≥1:4)
American Samoa	25	32
Fiji	181	26
New Hebrides	93	20
New Caledonia	193	18

Differences between individual islands in the New Hebrides (14, 14, and 36%) suggest that geographic variation may reward further study. We have attempted to use the rotavirus neutralization test of Thouless et al (1) to avoid the problems of anticomplementary sera and achieve results specific for human rotavirus. The test appears to work well with Nebraska calf diarrhea virus as the antigen. Sera from Fiji were 100% positive with a wide range of titers and lower titers in babies than in adults. Attempts to do the test with human rotavirus as the antigen have been frustrated by our inability to repeat the work of Thouless et al in which human rotavirus is centrifuged onto cell layers and produces antigen within 20 hours of growth.

A survey of stool specimens from newborns for rotavirus at Kapiolani and Kaiser Hospitals in Honolulu is currently in progress and will be completed by the end of the current contract expiration date. To date, in distinct contrast to results of Australian workers, no rotaviruses have been found in approximately 80 stools from newborns in 3 nurseries in Honolulu. Murphy et al (2) found that 49% of newborns in Sydney, Australia, were infected. Epidemiologic studies are needed to determine the reasons for this contrast.

Kawasaki Disease. From January to June 1978 an epidemic of Kawasaki Disease (mucocutaneous lymph node syndrome) has occurred in Honolulu. At least 26 cases have been identified, the highest incidence ever reported in the U.S. Complete epidemiologic and microbiologic studies are underway; the results will be reported at a later date.

References

1. Thouless, M. E., Bryden, A. S., Flewett, T. H., Woode, G. N., Bridger, J. C., et al. Arch Virol 53:287-294, 1977.

2. Murphy, A. M., Albrey, M. B., and Crewe, E. B. Lancet II:1149-1150, 1977.

See the following other projects given technical support from this project for additional results:

Z01-AI-00113-06 LPD
Z01-AI-00115-04 LPD
Z01-AI-00163-02 LPD
Z01-AI-00177-01 LPD
Z01-AI-00178-01 LPD

Proposed Course of the Project

The project will be terminated by October 1, 1978.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00177-01 LPD

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of the potential role of bedbugs as vectors of hepatitis B virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert B. Tesh, Staff Member, Pacific Research Section, LPD, NIAID

Other: Robert H. Purcell, Laboratory of Infectious Diseases, NIAID, NIH, Bethesda.
Leon Rosen, Head, Pacific Research Section, LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Pacific Research Section

INSTITUTE AND LOCATION

NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Laboratory-reared bedbugs (Cimex lectularius) were inoculated with infectious human sera containing each of the 3 known hepatitis B virus subtypes. The bugs were sampled at approximately 10 day intervals for 3½ months to determine how long hepatitis B surface antigen was detectable and if the titer increased. Results of this study are pending.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives

1. To investigate the possibility that bedbugs might serve as mechanical and/or biological vectors of hepatitis B virus.

Methods Employed

Bedbugs (C. lectularius) were reared in the laboratory by standard methods. The insects were tested individually for the presence of hepatitis B surface antigen (HBsAg) by radioimmunoassay.

Major Findings

Laboratory-reared C. lectularius were inoculated with infectious human sera (titrated in chimpanzees) containing each of the 3 known hepatitis B virus subtypes (adw, ayw and adr). The bugs were sampled immediately after inoculation and at approximately 10 day intervals for 3½ months. After trituration, the bugs were tested individually for the presence of hepatitis B surface antigen by radioimmunoassay. The initial experiments are now completed and we are awaiting the results.

Significance to Biomedical Research and the Program of the Institute

Although a great deal of knowledge about the epidemiology of hepatitis B has been gained in the past few years, the modes of its transmission are still uncertain. In developed regions of the world (i.e. northern Europe and North America) where infection with hepatitis B virus is relatively uncommon, transmission probably occurs mainly through inoculation of blood and blood products, although the virus may also be transmitted through other body fluids such as saliva, menstrual discharge, breast milk and semen. However, in some tropical areas of Africa and Asia where hepatitis B antigen carrier rates in the general population approach 10 to 20%, it is difficult to believe that inoculation of blood or blood products alone could account for the much higher infection rates.

One alternative mechanism for the transmission of hepatitis B virus might be through the bite of a blood sucking arthropod vector. Although this possibility has been suggested by a number of investigators before, most of the work to date has focused on the potential role of mosquitoes in the transmission of hepatitis B virus. HBsAg has been detected repeatedly in wild-caught mosquitoes, but there is little evidence that the virus replicates or even persists in these insects after digestion of the blood meal. In mosquitoes fed artificially on hepatitis B infected blood, the surface antigen usually disappears within 36-48 hours, suggesting that the virus and/or antigen are digested in the mosquito's stomach along with the serum proteins. Attempts to demonstrate hepatitis B virus transmission to chimpanzees by mosquitoes, after the latter had fed on infected blood, were also unsuccessful.

However, 4 recent studies suggest that the virus behaves quite differently in bedbugs and that these insects may serve as mechanical and/or biological vectors of hepatitis B virus. Naturally infected bedbugs (Cimex lectularius and C. hemipterus) have been collected from human dwellings in Africa on several occasions. In one study, 69% of C. hemipterus collected from the beds of HBsAg-positive persons were antigen-positive. Furthermore, the antigen was detectable in field-collected bedbugs kept alive without a blood meal for 30 days.

In another study, Newkirk et al. reported that detectable amounts of HBsAg were still present in C. lectularius 5 weeks after feeding on infected human blood. Initially, HBsAg titers in the bedbugs decreased, but by the fifth week there was an increase, suggesting possible replication of the virus and/or antigen. Unfortunately, this experiment was not carried beyond 5 weeks; however, assuming that hepatitis B virus is a relatively "slow virus", this finding is consistent with an extrinsic incubation period (time required for virus to complete development in the vector) as observed with other arthropod-borne viral agents.

In the past bedbugs were not considered vectors of human disease, but recently Cimicidae have been implicated as vectors and reservoirs of 3 arboviruses. Kaeng Khoi virus is presumed to be maintained in a bat-bedbug cycle in southeast Asia. Workers at the Center for Disease Control, Fort Collins, Colorado have shown that Oeciacus vicarius, which infests the nests of swallows in North America, is the biological vector and reservoir of Fort Morgan and Bijou Bridge viruses, antigenic members of the western and Venezuelan equine encephalitis serologic groups, respectively. Collectively, results of these studies suggest that bedbugs might serve as vectors of hepatitis B virus. The fact that they live in intimate contact with their human hosts and that they feed as often as 2 or 3 times a week further increases their vector potential.

Proposed Course of the Project

When the results of the forementioned experiments are available, the future course of the project will be determined. If hepatitis B virus replicates in bedbugs, then attempts will be made to demonstrate bite transmission of the virus to susceptible non-human primates. If the HBsAg persists but does not replicate, then the possibility of mechanical transmission must be considered. If the virus disappears within a few days and does not reappear, then it must be assumed that bedbugs are probably not important vectors of hepatitis B and the project will be discontinued.

Publications

None.

NOTE: Please see Project Number Z01 AI 00164-02 LPD for technical support provided.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00178-01 LPD

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of the mechanism of transovarial transmission of arboviruses by mosquitoes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert B. Tesh, Staff Member, Pacific Research Section, LPD, NIAID
Other: Leon Rosen, Head, Pacific Research Section, LPD, NIAID

COOPERATING UNITS (if any)

Yale Arbovirus Research Unit, Yale University College of Medicine, New Haven, Connecticut

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Pacific Research Section

INSTITUTE AND LOCATION

NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.7	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to identify those factors which control transovarial transmission of arboviruses by mosquitoes. Results to date indicate that a number of flaviviruses (Japanese encephalitis, yellow fever, dengue, Kunjin, Kokobera, Uganda-S, Ilheus, Banzi and Bussuquara) as well as two bunyaviruses (Keystone and San Angelo) can be transovarially transmitted by experimentally infected Aedes mosquitoes. In addition, differences were observed among several geographic strains of Aedes albopictus in their efficiency of vertical transmission of Kunjin virus. Different strains of Kunjin virus also varied in their ability to be transovarially transmitted in mosquitoes. These studies suggest that vertical transmission of arboviruses by mosquitoes may be an alternative mechanism for biological survival of the viruses.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives

1. To study the various factors (environmental as well as hereditary) which control transovarial transmission of arboviruses by mosquitoes.
2. To evaluate the importance of transovarial transmission in the maintenance of mosquito-borne viruses in nature.

Methods Employed

Mosquitoes were reared in an insectary by standard laboratory methods. The parent female mosquitoes were infected by direct intrathoracic inoculation of virus. After an appropriate incubation period, the infected mosquitoes were fed a blood meal and their subsequent eggs were collected and hatched. The F_1 larvae were reared to the adult stage and then were tested for the presence of virus by culture in tubes of Vero cells or by demonstration of specific viral antigen by direct fluorescent antibody technique.

Major Findings

A. Transovarial transmission of yellow fever virus

Adult female Aedes aegypti of the Santo Domingo, Kampala and Amphur geographic strains were infected by intrathoracic inoculation with yellow fever virus. The virus was subsequently recovered from F_1 adult offspring hatched from surface sterilized eggs laid by the infected parents, indicating that yellow fever virus was transmitted to the progeny through the egg. Infection rates in the F_1 offspring of the 3 A. aegypti strains were relatively low, in the range of 1:600. Both male and female progeny were infected. Interestingly, the virus was identified in the F_1 progeny from 3 different ovarian cycles and from eggs which had been stored at room temperature for several weeks. These findings suggest that vertical transmission of yellow fever virus in mosquitoes may be an alternative mechanism for biological survival of the virus during adverse climatic conditions or in the absence of susceptible vertebrate hosts.

B. Range of arboviruses which are transovarially transmitted in mosquitoes

Following our demonstration of vertical transmission of Japanese encephalitis and yellow fever viruses by mosquitoes, we decided to test a number of other arboviruses belonging to various serologic and taxonomic groups to determine if they might also be transovarially transmitted. The following viruses were tested in Aedes albopictus and/or Culex fatigans: dengue, West Nile, Ilheus, Kunjin, Kokobera, Uganda-S, Banzi, Bussuquara (flaviviruses); Sindbis, chikungunya, Semliki forest and Getah (alphaviruses); Hart Park and vesicular stomatitis, Indiana and New Jersey serotypes (rhabdoviruses); Keystone, San Angelo, Cache Valley and Turlock (bunyaviruses). Vertical trans-

mission was demonstrated in A. albopictus with dengue, Ilheus, Kunjin, Kokobera, Uganda-S, Banzi, Bussuquara, Keystone and San Angelo viruses. Filial infection rates with the 7 flaviviruses were relatively low, ranging from about 1:200 to >1:1,000, while those with Keystone and San Angelo viruses were 6:100 and 13:100, respectively. Our studies to date suggest that vertical transmission in mosquitoes occurs only with certain flaviviruses and bunyaviruses.

C. Additional studies with Kunjin virus

Kunjin virus was selected as a model for further studies of the factors controlling vertical transmission of flaviviruses in mosquitoes. Preliminary findings indicate: (1) that Kunjin virus is transovarially transmitted by A. albopictus but not by C. fatigans, (2) that there are differences among various strains of Kunjin virus in their ability to be transmitted transovarially, (3) that various geographic strains of A. albopictus differ in their efficiency (rates) of vertical transmission, and (4) that Kunjin virus can be sexually transmitted by infected males to non-infected females during copulation.

Significance to Biomedical Research and the Program of the Institute

One of the enigmas about the epidemiology of many mosquito-borne virus diseases is the mechanism by which the viruses survive during adverse climatic conditions (i.e. winter in temperate areas and dry season in tropical regions) when adult forms of the insect vector are absent or few in number. A variety of hypotheses have been proposed, none of which have been proven. However, recent experimental and field work indicates that some mosquito-borne viruses are transovarially transmitted and that this mechanism may permit the viruses to survive during adverse climatic conditions or in the absence of susceptible vertebrate hosts. Although this mechanism has previously been demonstrated with viruses transmitted by ticks and phlebotomine sandflies, until recently it was thought not to occur in mosquitoes. If transovarial transmission of arboviruses by mosquitoes is a widespread phenomenon in nature and not just a laboratory curiosity, then the epidemiology and control of such major human insect-borne diseases as Japanese encephalitis, dengue and yellow fever must be reconsidered.

Proposed Course of the Project

The Pacific Research Section will cease to be a part of the NIAID intramural program on October 1, 1978. Work on the current project will be continued under contract with the Research Corporation of the University of Hawaii.

Publications

Tesh, R. B., Saidi, S., Gaidamovich, S. Ya., Rodhain, F., and Vesenjak-Hirjan, J.: Serologic studies on the epidemiology of Phlebotomus fever in the Old World. Bull. WHO 54:663-674, 1976.

Tesh, R. B. and Wallace, G. D.: Observations on the natural history of encephalomyocarditis virus. Am. J. Trop. Med. Hyg. 27:133-143, 1978.

Tesh, R. B.: The prevalence of encephalomyocarditis virus neutralizing antibodies among various human populations. Am. J. Trop. Med. Hyg. 27:144-149, 1978.

Knudsen, A. B., Lewis, D. J., Tesh, R. B., Rudnick, A., Jeffery, J., and Singh, I.: Phlebotomine sandflies (Diptera: Psychodidae) from a primary hill forest in West Malaysia. J. Med. Entomol. (in press).

Tesh, R. B., Rosen, L., Beaty, B. J., and Aitken, T. H. G.: Studies of transovarial transmission of yellow fever and Japanese encephalitis viruses in Aedes mosquitoes and their implications for the epidemiology of dengue. Proc. Workshop on Dengue Fever in the Caribbean. PAHO Sci. Pub. (in press).

Tesh, R. B.: Arboviruses and their diseases - Central Asia and the Soviet Union. IN Feigin, R. D. and Cherry, J. D. (eds.): Pediatric Infectious Diseases. W. B. Saunders Co., Philadelphia (in press).

NOTE: Please see Project Number Z01 AI 00164-02 LPD for technical support provided.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00114-06 LPD

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on the arboviruses of the Phlebotomus fever serogroup

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. Robert B. Tesh, Staff Member, PRS,LPD,NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Pacific Research Section

INSTITUTE AND LOCATION

NIAID,NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been terminated.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00151-02 LPD

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Studies on the ecology of encephalomyocarditis virus.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. Robert B. Tesh, Staff Member, PRS,LPD,NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Pacific Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Honolulu, Hawaii

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been terminated.

PHS-6040
(Rev. 10-76)

LABORATORY OF IMMUNOLOGY
1978 Annual Report
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PHS-NIH
Summary Statement
Office of the Chief
Laboratory of Immunology
October 1, 1977 through September 30, 1978

Pre-B Cells Are Not Affected by Allotype Suppression

The earliest recognized member of the lineage of thymus-independent (B) lymphocytes is a cell which possesses cytoplasmic immunoglobulin but appears to lack membrane immunoglobulin. This cell is found in fetal tissues as well as in bone marrow of humans, mice and rabbits and is designated a pre-B cell. The pre-B cell appears to have already expressed a degree of immunologic commitment as in rabbits heterozygous at the b (κ chain) locus, an individual pre-B cell expresses detectable amounts of only one of the two alternative allelic forms present in the individual. This year, Laboratory of Immunology scientists, in collaboration with colleagues at the University of Alabama, have sought evidence concerning the functional potential of pre-B cells. They wished to determine whether such cells could be eliminated or "masked" by treatment of individual rabbits heterozygous at the b locus with antisera specific for one of the two allelic forms of b locus gene products possessed by the individual. Such treatment, termed allotype suppression, is known to eliminate immature B lymphocytes and appears to be a model for certain forms of tolerance induction. When neonatal rabbits which are b4/b5 in type are treated with anti-b5 serum, B lymphocytes expressing b5 are lost but there is no inhibition of b5 positive pre-B cells. This result strongly suggests that the pre-B cell, although differentiated in the sense of its commitment to the synthesis of a given type of immunoglobulin, is insensitive to modulation by anti-Ig antibody and is probably not susceptible to either tolerance induction or specific activation (Simons and Mage, LI/NIAID; Hayward, Cooper, Lawton, and Gathings, University of Alabama).

New Mouse Model For B Lymphocyte Immune Deficiency

One of the most powerful approaches to the dissection of the immune system of animals and humans has been the analysis of individuals with genetically determined immune defects. Scientists from the Laboratory of Immunology, together with colleagues in the Naval Medical Research Institute and the Laboratory of Microbial Immunity, have studied in detail the immune system of CBA/N mice, which have a mutant X chromosomal gene, xid, which in the homo- or hemizygous states leads to a profound but selective defect in B lymphocyte responses. During the past year, it has been shown that mice which are hemizygous for xid and which, in addition, possess a gene donated by C3H/HeJ mice, perhaps the gene regulating mitogen responses to lipopolysaccharide, they display much more serious immune defect than either parent. These mice make no antibody response to any thymus-independent antigen tested, are entirely unresponsive to B lymphocyte mitogens, have diminished numbers of B lymphocytes and have extremely low ratios of membrane IgD to membrane IgM. A congenic strain is being prepared by introgressive backcrossing of CBA/N mice to C3H/HeJ mice. It is anticipated that these mice will prove to lack essentially all mature B lymphocytes and may thus provide a valuable tool for the study of functions of

immature B lymphocytes (Bona, Mond and Paul, LI/NIAID; Scher, Naval Medical Research Institute).

B Lymphocyte Activation by Anti-Immunoglobulin Antibodies

The nature of the process by which B lymphocytes are activated by specific antigens is still largely undefined. One reason for this is that only a small fraction of the members of any B lymphocyte population are specific for a given individual antigen and thus the biochemical consequences of activation by antigen are difficult to study. Recently, Laboratory scientists have shown that specifically purified goat antibodies directed at mouse IgM are powerful stimulants of lymphocyte proliferation. This provides an alternative and more general approach to the study of lymphocyte activation since anti-IgM antibodies can interact with all B lymphocytes. Last year, it was shown that this response was not dependent on the Fc portion of the anti-IgM antibody nor were antibody aggregates required. This year, it has been shown that the responding cells are B lymphocytes and that the response is not dependent on T lymphocytes nor on the presence of macrophages. Furthermore, an analysis of the nature of the responding B lymphocytes has been made. These cells are absent from mice with the CBA/N immune defect, do not appear in mice until 4 weeks of age, and express larger than average amounts of total membrane immunoglobulin. Although anti-μ is a powerful stimulant of B lymphocyte proliferation, it does not, by itself, cause immunoglobulin synthesis. This suggests that interactions between globular proteins and B cell receptors, in the absence of T lymphocyte help induces B cell proliferation but do not cause differentiation to antibody production (Sieckmann, Mosier, Paul, LI/NIAID; Asofsky, LMI/NIAID; Scher, Naval Medical Research Institute).

Responses to TI-2 Antigens Are Inhibited by Anti-Lyb 7

Peripheral B lymphocytes of normal adult mice consist of two major subpopulations defined by an alloantibody to the B lymphocyte differentiation antigen Lyb 5. B cells which can be lysed by anti-Lyb 5 and complement (Lyb 5^+ B cells) are responsive to one class of thymus-independent antigens, the TI-2 antigens, of which trinitrophenyl-aminoethylcarbamylmethyl Ficoll (TNP-AECM-Ficoll) is the prototype. Lyb 5^- cells are not found in mice with the CBA/N immune defect; these mice are unresponsive to TI-2 antigens. Recently, it has been shown that in vitro anti-TNP antibody responses to TNP-AECM-Ficoll are inhibited by antiserum with anti-Lyb 5.1 activity, in the absence of complement. These sera do not block responses to TI-1 antigens, such as TNP-Brucella abortus, to which mice with the CBA/N defect are responsive. Through the analysis of backcross progeny of the mating of (C57BL/6 x DBA/2)F_1 x C57BL/6 mice, it can be shown that the ability of anti-Lyb 5.1 antisera to lyse cells in the presence of complement and the ability to block in vitro B lymphocyte responses to TNP-AECM-Ficoll are each controlled by a single gene but these genes are not linked to each other. The antigen for which the "blocking" antibody is specific has been designated Lyb 7.1. Genetic analysis indicates that the gene for Lyb 7.1 is 15 to 20 centimorgans from the genes which specify immunoglobulin H chain constant regions. This linkage between genes for membrane immunoglobulin receptors and the gene for an alloantigen which regulates B lymphocyte activation may reflect an underlying functional association of the gene products and may provide insight into the mechanism by which anti-Lyb 7.1 inhibits certain

B cell responses (Subbarao, Mosier, and Paul, LI/NIAID; Ahmed and Scher, Naval Medical Research Institute).

Suppression of Antibody Responses by Anti-Idiotype Antibodies

One of the more challenging problems in the understanding of the control of the immune response is to delineate the mechanism by which the emergence of various individual clones of antibody secreting cells are regulated. A powerful approach to the study of such regulation has been to examine the appearance of cells bearing receptors, and capable of secreting antibodies, which express idiotypic determinants shared by other receptors and antibodies of the same specificity. Laboratory scientists have played an important role in the delineation of two such systems of cross-reactive idiotypes IdX's, those involving antibodies specific for bacterial levan (BL) and phosphorylcholine (PC). The IdX characteristic of the BL system is the E109 IdX; that for the PC system is the T15 IdX.

During the past year two groups of laboratory scientists have studied the regulation of expression of IdX by pretreating mice with anti-IdX antisera prior to immunization. In the BL system, pretreatment with anti-E109 antisera prior to immunization with BL leads to a complete and prolonged suppression of those clones of anti-BL antibody secreting cells whose products express E109. This process is thymus-indpendent and appears to result from the direct contact of anti-E109 with the precursor of an E109 bearing cell (Bona, Lieberman, Green and Paul, LI/NIAID).

In the PC system, treatment of mice with anti-T15 antibody prior to immunization with a protein, such as hemocyanin, leads to the development of T lymphocytes which specifically suppress the secondary response of PC primed B cells to PC-hemocyanin. These suppressor $\overset{-}{T}$ cells appear to be both idiotype and carrier specific and express the Ly 1^{-} 2^{+} phenotype. Such cells may play a critical role in normal control of B lymphocyte responses (Bottomly and Mosier, LI/NIAID; Mathieson, LMI/NIAID.

Structure of Guinea Pig Ia Antigens

All mammalian species thus far studied express a family of membrane glycoproteins coded for by genes in the I-region of the major histocompatibility complex. These molecules are referred to as Ia antigens. Ia antigens have important roles in the regulation of interactions between immunocompetent cells, in the function of specific immune response (Ir) genes, and in the stimulation of mixed lymphocyte responses. Because of these major functions, structural data on Ia antigens are extremely important. As previously described, there appear to be three distinct types of guinea pig Ia antigens, two of which have been shown to contain two types of polypeptide chains, α and β. These chains are linked by disulfide bonds in one instance and by non-covalent bonds in another. Analysis of the N terminal amino acid sequence of the lighter (β) polypeptide chain indicates a substantial homology between all guinea pig β chains. This homology is greater when one compares β chains derived from allogeneic forms of the disulfide linked Ia antigen than when one compares the β chains from disulfide bonded Ia antigens of a given strain with the β chains

of the non-covalently bound Ia antigen of the same strain. Furthermore, peptide mapping of the non-disulfide bonded Ia antigen from strain 2 guinea pigs suggests a very substantial homology between the α and β polypeptide chains. This is consistent with the α and β chain being derived from a single gene and then being separately processed so that the β chain is a fragment of the α chain. Alternatively, the α and β chains may be encoded by recently re-duplicated genes which are still closely associated with each other. Further studies to more completely assess the structure of Ia antigens and to correlate their structure with their functions are now in progress (Waxdal, Tyran, Clement and Shevach, LI/NIAID).

Both Macrohage-T Lymphocyte and T Lymphocyte-B Lymphocyte Interactions are Restricted by I-region Gene Products.

Laboratory of Immunology scientists have previously demonstrated that the in vitro activation of primed T lymphocytes requires that the T lymphocyte recognize both antigen and I-region gene products expressed on an antigen-presenting cell. In F_1 guinea pigs immunized to an antigen such as ovalbumin (OVA), two independent sets of OVA-specific T lymphocytes can be demonstrated - one which responds to OVA presented by cells from one parent, but not the other and a second with the opposite responsiveness pattern. If recognition of antigen and I-region gene products was a general characteristic of T lymphocyte activation, it would be anticipated that interactions between T and B lymphocytes would show a similar restriction and that those F_1 T lymphocytes which interact with antigen-presenting cells from one parent but not the other would express the same specificity in their interaction with B lymphocytes. Primed T lymphocytes were obtained from immunized F_1 guinea pigs and were selected by culture for one week with antigen-presenting cells derived from one parent. At the end of the week it could be shown that the only remaining antigen-responsive cells were those which could be activated by antigen-presenting cells of the type used in the initial culture. When tested for helper activity using B lymphocytes from either parent, the "selected" T cells could collaborate only with B lymphocytes derived from the parent which was of the same genotype as the antigen-presenting cells used in the initial culture. These results indicates that there is a definite symmetry in the interactions of T lymphocytes with antigen-presenting cells and with B cells. It suggests that recognition of both antigen and Ia is a general rule in T cell responses (Yamashita and Shevach, LI/NIAID).

The Antigen-Presenting Cell of the Mouse is an Ia^+, $Thy\ 1^-$, Ig^-, Radioresistant, Adherent Mouse Spleen is Major Antigen-Presenting Cell.

The in vitro activities of most T lymphocytes requires presentation of antigen by a live cell which is believed to be a member of the monocyte-macrophage lineage. Despite the important role of this cell, it had not heretofore been carefully characterized. During the past year, Laboratory scientists defined several important aspects of this cell. Antigen-presenting cells were found to be quite abundant in the spleen but surprisingly, peritoneal exudates and bone marrow seem to be quite poor in antigen-presenting activity. The antigen-presenting spleen cell is adherent to glass and plastic surfaces and is resistant to radiation. It is destroyed by treatment with antisera to either

I-A or I-E/C antigens together with complement (C). Neither anti-Ig nor anti-Thy 1 sera and C diminish the antigen-presenting activity of spleen cells. This result suggests that at least one type of antigen-presenting cells has the character that it is an adherent, radioresistant I-A and I-E/C positive cell which lacks Thy 1 and Ig. It has not yet been established whether this cell is actively phagocytic. It seems most likely that antigen-presenting cells contribute a distinct subset or a distinct differentiated state of the macrophage lineage.

Additional studies carried out this year have reinforced the concept that specific immune response (Ir) genes are expressed within antigen-presenting cells. This was shown by demonstrating that T lymphocytes from primed F_1 mice which were derived from crosses between responder and non-responder mice could be activated by antigen presented on spleen cells from the responder parent but not by antigen presented on spleen cells from the non-responder parent. A series of approaches were used to demonstrate that the failure of non-responder antigen-presenting cells to function in this system was not due to the generation of suppressor T cells. These results emphasize the critical role of antigen-presenting cells in T lymphocyte activation and suggest approaches to the enrichment of these cells so that they can be carefully studied. (Schwartz, Yano, and Paul, LI/NIAID).

The Langerhans Cell is an Ia^+ Antigen-Presenting Cell Found in the Skin

Recently, scientists working in the Laboratory of Immunology and in the Dermatology Branch of the National Cancer Institute had demonstrated that the principal cell in the guinea pig skin which possessed Ia antigens was the Langerhans cells. This is a macrophage-like cell which constitutes only a small fraction ($\sim$ 3%) of skin cells. Since previous Laboratory of Immunology studies indicated that cells in lymphoid tissues capable of activating or presenting antigen to T cells were all Ia positive, it was suggested that the Langerhans cell might be critical in immunization processes mediated through the skin, such as contact hypersensitivity and in sensitization of recipients for the rejection of skin allografts. During the past year the antigen-presenting functions of skin cell populations enriched in or depleted of Langerhans cells were studied. It was shown that Langerhans cell enriched population were equal to macrophages as antigen-presenting cells while skin cell populations depleted of Langerhans cells have essentially no antigen-presenting activity. This provides a functional correlate of the previous studies of Langerhans cell surface antigens. It reinforces the concept that the "immunogenic" potential of the spleen is principally a function of a minor subpopulation and raises the possibility that elimination of such cells from transferred allografts might markedly prolong graft acceptance (Shevach and Green, LI/NIAID; Stingl and Katz, Dermatology Branch, NCI).

The Immune Response to a Single Identified Antigenic Determinant in Cytochrome c is Controlled by Two Complementing Ir Genes

The cytochrome c's constitute a family of molecules which are exceptionally well suited for detailed study of the chemical nature of protein antigen determinants and sites of action of Ir gene products. There are more than 85

well characterized evolutionary variants of cytochrome c providing alternative residues at many of the amino acid positions of the molecule. The overall structure of cytochrome c is extremely well preserved and is quite precisely known as a result of x-ray crystalographic studies. The immune response to pigeon cytochrome c requires the presence of responder alleles of two distinct Ir genes. By analysis of the capacity of related cytochrome c's to stimulate proliferative responses in T lymphocyte populations from donors primed to pigeon cytochrome c, it could be shown that the amino acid substitutions leading to reactivity were at positions 100 and 104. Furthermore, individual variants sharing only positions 100 or 104 with pigeon cytochrome c gave some cross reaction; both appeared to stimulate the same set of specific T cells, indicating that only a single antigenic site existed, contributed to by both positions 100 and 104. This result indicates that the action of the two distinct Ir genes involved in the response to pigeon cytochrome c cannot be the recognition of distinct determinants. Rather, products of the two Ir genes must act in a concerted fashion to allow the recognition of a single antigenic determinant probably in a single cell (Schwartz and Solinger, LI/NIAID; Ultee and Margoliash, Northwestern University).

Patients with Systemic Lupus Erythematosus Have a Defect in the "Auto" Mixed Lymphocyte Response

It is well known that mixing lymphoid cells from genetically distinct individuals leads to a striking proliferative response. This mixed lymphocyte response (MLR) is principally due to the recognition by T lymphocytes of allogeneic histocompatibility antigens expressed by either (or both) B lymphocytes and macrophages. Recently, it has been shown that mixing purified human T lymphocytes with non-T lymphocytes from the same individual also leads to a substantial degree of proliferation. This response has been termed the auto-MLR. During the past year, it has been shown that patients with active systemic lupus erythematosus (SLE) display little or no auto-MLR cell. Mixing experiments suggest that the principal defective cell in the SLE patient is a T lymphocyte which bears Fc_{γ} receptors. Although the significance of the auto-MLR has not yet been established, it may reflect a normal self-recognition or immunoregulatory mechanism. The fact that patients with SLE are deficient in this activity is consistent with their profound defects in immunoregulation (Sakane and Green, LI/NIAID; Steinberg, Arthritis and Rheumatism Branch, NIAMDD).

Administrative, Organization and Other Changes

The Laboratory of Immunology continued to play an important role in training young investigators. During the past year, two research associates, Dr. Alan Solinger and Dr. James Mond, completed their training in the Laboratory of Immunology as did Drs. Cheng-po Hu, Uki Yamashita, and Akihiko Yano, who were Guest Workers. Each of these scientists made important research contributions during their tenure in the Laboratory of Immunology.

Drs. Herbert Herscowitz, Dan Longo, Louis Matis, Patricia Mongini and Oli Werdelin entered the Laboratory as Guest Workers and Drs. Bronislaw Tyran and Franco Meloni were appointed to Visiting Fellowships.

Honors, Awards, and Scientific Recognition

Laboratory scientists play important roles in the national and international scientific community. Many serve on the editorial boards of important journals. During the past year, Dr. Paul completed a four year term as section editor for cellular immunology of the Journal of Immunology. Drs. Green, Mage, Mosier and Shevach are associate editors of this journal. Dr. Mage is chairman of the editorial board of Federation Proceedings. She and Dr. Paul are members of the editorial board of the Journal of Immunologic Methods. Dr. Paul and Dr. Shevach are referee editors of the Proceedings of the Society for Experimental Biology and Medicine. In addition, Dr. Paul is an associate editor of the Journal of Experimental Medicine, Immunological Reviews, the Scandinavian Journal of Immunology, Immunologic Communications, and CRC Critical Reviews in Immunology. Dr. Green is a member of the editorial board of Clinical Immunology and Immunopathology; Ms. Lieberman is an advisory editor of Immunochemistry; and Dr. Shevach is a member of the editorial advisory board of the FASEB Handbook on Inbred Strains of Laboratory Animals.

Dr. Paul was the principal organizer of the Fourth Ir Gene Workshop in Annapolis, MD. and he and Drs. Green, Schwartz and Shevach served on the program committee for the Workshop. Dr. Paul presented invited lectures at the Midwinter Conference of Immunologists, at the Sixth International Convocation of Immunology, at the Arden House Conference on B Lymphocytes, at the meeting in honor of the 100th anniversary of the founding of the William Welch Pathology Laboratory, and at the XII Leucocyte Culture Conference. He was a Visiting Professor at the Hebrew University, Jerusalem, Israel and co-directed the Cold Spring Harbor Course on Immunogenetics. Dr. Paul is a Counselor of the American Society for Clinical Investigation, a member of the Fellowship Subcommittee of the Arthritis Foundation, of the Committee on Personnel for Research of the American Cancer Society, and of the Program Committee of the American Association of Immunologists.

Dr. Mage was a principal organizer of the International Workshop on Immunogenetics of the Rabbit, held in Bethesda, MD from May 3-5, 1978. She organized the joint Johns Hopkins-NIH Immunobiology Program, and gave a group of lectures at the Universities of Alberta and Manitoba. She was an invited lecturer in the Cold Spring Harbor Immunogenetics Course.

Dr. Green spent one month at the Medical Research Council Laboratory in the Gambia conducting field studies on the feasibility of a malaria vaccine. He was an invited speaker at the Sixth International Convocation on Immunology and at the meeting in honor of the 100th anniversary of the founding of the William Welch Pathology Laboratory. He is a member of the Transplantation and Immunology Advisory Committee, NIAID and is a consultant to the American Academy of Microbiology.

Dr. Shevach presented major lectures at the annual meetings of the American Society for Microbiology and the Canadian Society for Immunology. He was a workshop chairman at the XII Leucocyte Culture Conference and a lecturer at the Cold Spring Harbor Immunogenetics Course. He is a member of the Allergy and Immunology Study Section of the Division of Research Grants and is a

member of the External Advisory Committee of the Multiple Sclerosis Center at the University of Pennsylvania. Dr. Shevach was awarded a USPHS Commendation Medal this year.

Dr. Mosier presented invited lectures at the Symposium on Developmental Immunology, Aberdeen, Scotland and at the Strasburger Symposium at Cornell Medical College. He was a WHO Lecturer in Immunology in São Paulo, Brazil.

Dr. Waxdal was an invited lecturer at the Conference on Complex Carbohydrates in Biological Recognition. Ms. Lieberman gave an invited presentation at the Homogenous Immunoglobulin Workshop and Dr. Inman was a recipient of the NIH Director's Award. In addition, laboratory scientists presented research seminars at major universities and research institutes in the United States and abroad.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00030-10 LI

PERIOD COVERED

October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Antigen Recognition and Activation of Immunocompetent Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: William E. Paul, Chief, LI/NIAID

Other Investigators: Donald E. Mosier, Sr. Investigator, LI/NIAID
Rose Lieberman, Sr. Investigator, LI/NIAID
James J. Mond, Research Associate, LI/NIAID
Donna G. Sieckmann, Guest Worker, LI/NIAID
Kathryn Stein, Guest Worker, LI/NIAID
Toby Hecht, Senior Staff Fellow, LI/NIAID
Constantin Bona, Guest Worker, LI/NIAID
Richard Asofsky, Chief, LMI/NIAID
Irwin Scher, Naval Medical Research Institute
Aftab Ahmed, Naval Medical Research Institute

COOPERATING UNITS (if any)

LMI/NIAID; Naval Medical Research Institute

LAB/BRANCH

Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The general goal of this project is to delineate the mechanisms involved in antigen recognition by and activation of thymus-independent (B) and thymus-dependent (T) lymphocytes. During the past year our efforts have focused on: 1) the analysis of immunologic function of mutant mice with one or more defects in B lymphocyte function; 2) the analysis of the activation of B lymphocytes by anti-immunoglobulin antibody; 3) the role of non-immunoglobulin membrane alloantigens in B lymphocyte activation. In addition, a research program on the nature of the association membrane budding viruses with histocompatibility antigens has been initiated in order to gain further insight into biologic functions of histocompatibility gene products.

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(Rev. 10-76)

Our principal research interests in the past year have concentrated on an analysis of development and heterogeneity of B lymphocytes and on the regulation of B lymphocyte activation. We have assessed the expression of membrane Ia antigens as an index of immunologic maturity of antigen-responsive cells. It had been previously reported by others that precursors capable of giving primary IgM antibody responses to thymus-dependent antigens lacked Ia antigen on their surface. We evaluated the presence of Ia antigens on B lymphocytes which can respond to thymus-independent (TI) antigens. Our results indicate that B lymphocytes that respond to both of the principal classes of TI antigens (TI-1 and TI-2 antigens) express Ia antigens, including products of both the I-A and I-E/C regions. Ia antigens were found on precursors of IgM secreting cells as well as precursors of IgG secreting cells. Furthermore, precursors of antibody secreting cells present in two day old mice express Ia antigens. These results indicate that all cells capable of responding to TI antigens have already expressed Ia antigens and suggests that immunologic responsiveness may depend upon the expression of Ia antigens by immunocompetent cells.

A second group of studies aimed at defining developmental characteristics of B lymphocytes has involved an analysis of immune responsiveness of immunologically defective CBA/N mice. These mice possess a mutant gene, xid, which is located on the X chromosome between the tabby and hypophosphatemia genes. When xid is present in a homozygous or hemizygous form, the mice express a profound immunologic defect due to the absence of a subset of B lymphocytes which bear the phenotypic marker Lyb5. During the last several years we have characterized many of the defects of these mice in an effort to understand the various functions of $Lyb5^+$ and $Lyb5^-$ cells. During the past year, we have analyzed the immune responses of mice with the CBA/N defect to certain classes of antigenic determinants. Our results indicate that these mice make deficient responses to phosphoryl choline determinants and to $\beta 2 \rightarrow 6$ fructosan determinants even when these antigens are presented on very immunogenic carriers. Furthermore, when immunized with isomaltotriose (IM-3) conjugates of hemocyanin, mice with CBA/N defect make anti-IM3 antibodies which cross react poorly with the $\alpha 1$-6 determinants found in dextran in contrast to the response of normal animals in which there is an excellent cross reaction between IM-3 determinants and determinants present in $\alpha 1$-6 dextran. Two major alternative explanations of these results are: 1) The library of V_H genes expressed in $Lyb5^+$ and $Lyb5^-$ B lymphocytes is different and responses of these individual B lymphocyte classes will thus differ in specificity; 2) The susceptibility to tolerance induction of $Lyb5^+$ and $Lyb5^-$ cells is different and the failure of $Lyb5^-$ cells to make antibody responses of a given specificity is due to tolerance induction by cross-reactive environmental antigens. Efforts to test both theories will be made during the coming year.

Because of the great value of genetically defective mice in the analysis of the immune response, we have sought additional immunodeficiency models. One approach has involved the mating of CBA/N mice with C3H/HeJ mice which have an autosomal genetic defect rendering them unresponsive to lipopolysaccharide. We prepared F_1 hybrids from CBA/N female mice and C3H/HeJ males

and crossed them to C3H/HeJ males. Approximately half of the male backcross progeny expressed a very severe immunologic defect, much more profound than that of either of the original parents. The defect involves unresponsiveness to all B lymphocyte mitogens and to all thymus-independent antigens, diminished numbers of B lymphocytes and very diminished amounts of mIgD per B cell. Our genetic analysis suggests that these profoundly immunodeficient animals are hemizygous for the xid gene and heterozygous or homozygous for the deficient LPS gene. We are extending our functional characterization of these mice in the hope that they will provide a model to study functions of primitive B lymphocytes. In addition, we are in the fourth backcross generation in the production of a congenic strain possessing this combined immunodeficiency.

Our studies of the regulation of B lymphocyte activation have centered on the role of specifically purified anti-μ antibodies in initiation of proliferative responses. We have shown that $F(ab')_2$ fragments of purified goat anti-mouse μ antibodies will cause proliferation of mouse spleen cells. During the past year, we have demonstrated that this is a response of B lymphocytes and that it does not depend either on T lymphocyte or macrophage activity. Furthermore, we have isolated B lymphocyte subsets based on density of mIg or presence of Lyb5. Our results indicate that B lymphocytes which are $Lyb5^+$ and which bear large amounts of mIg are the principal cells responsive to anti-μ. We are currently attempting to find conditions which will allow anti-μ to stimulate Ig synthesis as well as proliferation .

During the past year we have initiated a new research program based on the analysis of the interaction of viruses such as vesicular stomatitis virus (VSV) and histocompatibility antigens. Previous work had shown that VSV incorporated histocompatibility antigens within its membrane during its growth within cells and that VSV grown in cells of a given histocompatibility type would preferentiably associate with histocompatibility antigens of the same type. We are in the process of analyzing these events both by serologic and chemical approaches and determining whether the association may have any immunologic significance.

In addition, we have established a facility for somatic cell hybridization and hybridoma production in order to take advantage of new technology for production of homogeneous antibodies.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00148-03 LI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Lymphocyte Interactions, Receptors and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other Investigators: Helen Hu, LI/NIAID
Tsuyoshi Sakane, LI/NIAID
Alfred Steinberg, Arthritis and Rheumatism Branch, NIAMDD, NIH

COOPERATING UNITS (if any)

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Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

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2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of the L_2C lymphatic leukemia of inbred strain 2 guinea pigs - efforts are now being focused on the chemical nature of a soluble KCL extract containing a tumor specific transplantation antigen (TSTA) obtained from these cells. The active moiety has a M.W. of 20,000 and is basic. It does not appear to be related to the cell surface IgM. The material is heat resistant but is sensitive to trypsin, neuraminidase and periodate. These properties suggest that the TSTA is a low M.W. glycoprotein.

We are investigating human suppressor T cells generated by concanavalin A activation. Such suppressor T cell activity is diminished in most patients with systemic lupus erythematosus (SLE). In addition, we have observed that in most patients with active SLE that the autologous mixed lymphocyte reaction (auto MLR) between T cells and non-T cells is also absent. The T cell responsible for auto MLR and that which mediates suppressor function may be one and the same cell.

PHS-6040
(Rev. 10-76)

The Guinea Pig L_2C Leukemia

The L_2C lymphatic leukemia of inbred strain 2 guinea pigs arose about 25 years ago in an old female strain 2 guinea pig. Studies in our laboratory starting 8 years ago have shown that this leukemia cell is of B cell origin and has surface IgM and C3 receptors. There are several varients of this leukemia; one varient exists which lacks Ia antigens (an antigen of the major histocompatibility complex of the guinea pig). Our laboratory has demonstrated that, as measured by immunization protection tests in inbred strain 2 guinea pigs, the L_2C cell has a powerful tumor specific transplantation antigen (TSTA). However, in the Ia negative varient of the L_2C cell this TSTA is not immunogenic.

Alloantisera can be prepared against the L_2C cell in strain 13 guinea pigs. This alloantisera contains both anti-Ia antibodies and antibodies to an idiotypic determinant of the cell surface IgM. The anti-Ia antibodies can be removed by absorbtion with normal strain 2 lymphocytes. The resultant anti-idiotypic sera does not react with any Ia like molecule. More recently, a syngeneic serum has been prepared to L_2C cells and to a KCL extract prepared from L_2C cells. This syngeneic antiserum recognizes the same IgM idiotypic determinant as was recognized by the alloantisera. This was determined by sequential immune precipitation and polyacrylamide gel electrophoresis. Such idiotypic determinants are not found on the IgM molecules of normal strain 2 lymphocytes. Thus, the idiotypic determinant of L_2C IgM molecules are tumor associated antigens (TAA).

The main thrust of our investigations of the L_2C leukemia during the past 2 years has been an investigation of the relationship between the TAA (the IgM idiotype) and the operationally defined TSTA. Our present studies in this regard are principally concerned with the immunogenic TSTA in a KCl extract prepared from the L_2C cell.

Immunization of strain 2 guinea pigs with 250 µg of the KCl extract completely protects animals against a subsequent challenge with a lethal dose of L_2C cells. Extracts of L_2C cells prepared by different procedures, such as simple cell supernatants, NP-40 extracts, papain digests and Triton X-100 soluble extracts were either less effective or not effective in immunization. The specificity of the KCl L_2C extract was demonstrated by the fact that similar extracts prepared from line 10 hepatoma cells (from strain 2 guinea pigs) were completely ineffective. Ammonium sulfate fractionation of L_2C extracts showed the immunogenic material to be present in the supernatant of 67% saturated fraction. This latter data, together with the fact that gel filtration of the KCl extract on G-200 Sephadex showed the active material to be in a fraction of M.W. less than 20,000 daltons, indicated that the TSTA was of rather low M.W. Since determinations of the quantity of the IgM idiotype had previously demonstrated that the non-immunogenic papain digest had considerably more IgM than did the immunogenic KCl extract and since the M.W. of the TSTA was less than 20,000 daltons, it can be almost certainly concluded that the TSTA is not an IgM molecule.

Our latest studies of the immunogenic component of the KCl extract demonstrate that it is resistant to heat, (boiling for 5 minutes does not inactivate it) and extremes of pH. However, it is sensititve to trypsin, neuramindase and periodate oxidation. The properties suggest that it is a low M.W. glycoprotein. Assay of the immunogenicity of DEAE cellulose chromatographic fractions at pH 8.4 suggested that the material was basic. Further purification by CM-Sephadex and isolectric focusing is now in progress.

Significance

The apparent M.W. of the TST of the guinea pig L_2C leukemia is considerably lower than the TSTA of any other recently described moiety from either animal or human tumors. A more complete knowledge of the nature of TSTA will eventually be necessary to design rational immunotheraputic approaches for cancer therapy. The fact that this TSTA is on a malignant B lymphocyte is of particular interest; once the L_2C TSTA is more completely identified one could look for a similar TSTA in several different human B cell leukemias. Another possibility is that this TSTA could represent a marker for a particular stage of B cell differentiation present on only a very small number of normal B cells.

Human Suppressor T Cells in Health and Disease

The second aspect of our studies concerns suppressor cells in patients with Systemic Lupus Erythematosus (SLE). The system we are using is adapted from Shou et al., J. Exp. Med. 143, 1100, 1976. The use of this system to describe Concanavalin induced suppressor cells in normal individuals has already been published by us in the J. of Immunology 119, 1169, 1977.

Briefly, a two stage assay system is employed in which T lymphocytes are exposed to concanavalin A (Con A) for 3 days to induce suppressor function and then these cells, after mitomycin C treatment, are added to an assay system consisting of lymphoid cells obtained from the same donor 3 days later. As a control, another aliquot of cells incubated without Con A is used. These assay cultures are then stimulated by either mitogen or allogeneic cells, cells from the first culture are added and the degree of proliferation is measured by ^{3}H-thymidine incorporation.

We have now used this assay of suppressor cells in 10 patients with active SLE. The majority of these patients showed some defect in the development of suppressor cells. Mixing experiments, between normal T cells and SLE T cells as either potential suppressor cells or as cells in the assay cultures, reveal that the defect in suppressor phenomenon in the cells from patients with SLE is in the actual generation of suppressor cells rather than in reception of a suppressor message.

Studies have now been performed in which sera from SLE patients have been added to cultures of normal lymphocytes during the Con A mediated induction of suppressor cells. SLE sera but not normal sera block the development of suppressor cells. Furthermore, heat inactivation of the SLE sera destroys this blocking effect. Fractionation studies show, that in the SLE sera, it is the IgM fraction and not the IgG fraction which blocks the formation of suppressor cells. Thus our current evidence supports the idea that the IgM fraction of certain SLE sera is cytotoxic for a subfraction of T cells which normally act as suppressor cells.

In the course of the above studies, another very interesting observation has been made. That is T cells and non-T cells, derived from normal individuals, produces a proliferative response. This proliferation response has been called the autologous mixed lymphocyte reaction (auto MLR). The T cell is the responder cell and non-T cell is the stimulator cell in this reaction. Others have shown that the auto MLR is probably an immunological response because it has specificity and memory. Although the reason for the auto MLR is not understood, one current speculation is that it has some type of immuno-regulatory role. The accidental observation that was made was that this auto MLR is entirely absent in patients with active SLE and is diminished in patients with inactive SLE. In contrast, the ordinary allogeneic MLR is normal when lymphocytes from patients with SLE are used as either responder or stimulator cells.

Further studies of this defective MLR in patients with SLE strongly suggest that the defect is in the responder T cell population rather than in the stimulator cell population. Moreover, studies of T cells fractionated into IgG Fc^+ and IgG Fc^+ classes indicate that the defect is in the Fc^+ class. Of great interest is the fact that in a separate system of assaying suppressor cell activity, Moretta et al. have shown that an IgG Fc^+ T cell is also a suppressor cell. Partially stimulated by this observation, we did a simultaneous study of suppressor cell and auto MLR activity in 6 SLE patients and 6 normal individuals. In each instance the lymphocytes of patients with SLE, but not the normal lymphocytes, expressed a defect in both Con A induced suppressor cells and in the autologous MLR.

Significance

Since the abnormal auto-antibody production observed in patients with SLE (which is responsible for many of the disease manifestation) may result from the relative absence of suppressor cells and defective autologous MLR, an understanding of these relationships and identification and synthesis of soluble suppressor materials may provide a basis for the treatment of patients with SLE. Such a strategy using a Con A induced soluble suppressor material (SIRS) in mice has already been used to successfully treat mice with SLE.

Our further studies in this area will be directed to our analysis of the effects of various SLE sera on both suppressor cells and cells responsible for autologous MLR. It would be most important to determine if these are indeed the same cells and whether they both possess Fc^+ receptors.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00034-06 LI

PERIOD COVERED

October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Differentiation and Activation of Mouse T and B Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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William E. Paul, M.D., Chief, LI/NIAID

COOPERATING UNITS (if any) Irwin Scher, M.D., Staff, Naval Medical Research Institute; Bonnie Mathieson, Ph. D., Staff Associate, LMI/NIAID; R.A. Asofsky, M.D., Chief, LMI/NIAID; A. Ahmed, Ph. D., Naval Med. Res. Assoc.

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Laboratory of Immunology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The differentiation of T and B lymphocyte subpopulations, both before and after stimulation with T-dependent or T-independent antigens, is being studied. Particular attention has been paid to the state of reactivity of neonatal mouse lymphocytes. Two major B cell sublines have been described based upon their reactivity to antigen, surface immunoglobulin expression (surface IgM > surface IgD, and vica versa), expression of the Lyb5 differentiation antigen, and presence in the CBA/N mutant subline mouse. Some T-independent antigens have been found to cause priming for enhanced antibody responses following injection into newborn mice; this priming results from antigen interacting only with one immature B lymphocyte subpopulation.

PHS-6040
(Rev. 10-76)

The general long-range objectives of the work in this laboratory are to define functional subpopulations of murine lymphocytes, describe their surface properties, and understand how antigen induces lymphocyte activation and which cell surface components are involved in activation. We are concerned particularly with the emergence of lymphocyte subpopulations during ontogeny and how their interaction affects the total immunological competence of the newborn.

The methodology employed in addressing these problems involves: (1) Identification and separation of lymphocyte subpopulations based primarily on differential expression of cell surface components such as surface immunoglobulin or Lyb5 differentiation antigens. Specific antisera prepared against such surface components allow either complement-dependent lysis, separation of fluorescent antibody labeled cells on the fluorescence-activated cell sorter (FACS), or blockade of function by incorporation of the antisera into tissue culture; (2) The ability to culture small numbers of purified lymphocyte subpopulations (2×10^4 or greater) in microcultures and stimulate proliferation or antibody formation is crucial to these experiments and has been established; (3) The analysis of mice with genetic defects affecting B lymphocytes, such as the CBA/N or C57BL/10 ScCr strain, has proven to be a powerful tool in analyzing correlations between membrane structure and B cell function; (4) The preparation of anti-idiotypic antisera has allowed the investigation of the network regulation of immune responses of T and B lymphocytes.

The major findings to emerge from these studies are as follows: Studies of B cell functional differentiation in the neonatal mouse have led to the description of the following maturational sequence. The first set of B cells to develop after birth is characterized by: (1) high surface immunoglobulin (sIg) density; (2) sIgM > sIgD; (3) absence of the Lyb5 antigens(s); (4) responsiveness to TNP (trinitrophenyl)-Brucella abortus and a related series of antigens; and (5) unresponsiveness to TNP-Ficoll and other polysaccharide antigens. A later appearing B cell subset is characterized by: 1) low sIg density, 2) sIgM < sIgD, 3) presence of the Lyb5 marker, 4) absence in the genetically-deficient CBA/N strain, and 5) responsiveness to a broader range of thymic-independent antigens typified by TNP-Ficoll. In addition, the late-appearing B cell subset is susceptible to inhibition of its antibody responses by allo-anti-δ reagents, whereas the earlier appearing subset seems resistant to such inhibition. These studies thus have led to the definition of two major B cell subsets with both different markers and different functions.

A genetic analysis of the expression of the Lyb5.1 alloantigen has been undertaken. Two activities of anti-Lyb 5.1 were assessed in (C57BL/6 x DBA/2) x C57BL/6 backcross mice. One activity was the ability to kill about 50% of spleen cells of Lyb 5.1^+ strains in the presence of complement (=cytotoxic activity). The second activity was the ability to inhibit selectively the in vitro antibody response to TNP-Ficoll (=blocking activity). Both the antigen responsible for cytotoxic activity and the antigen responsible for

blocking activity were encoded by a single locus; however, the two loci segregated independently in the backcross animals. A new antigen, tentatively designated Lyb7.1, is therefore recognized by anti-Lyb5 antiserum. The expression of this antigen is not linked to the H-2 or Mls regions, but is linked to the allotype (C_H) locus.

A final series of experiments is concerned with the mechanism of clonal dominance in the antibody response of BALB/c mice to phosphorycholine (PC). Both T and B cells seem to express a common dominant idiotype on their immunoglobulin (-like) receptors; this idiotype is termed T-15, after the prototype TEPC-15 myeloma protein. Anti-T15 antisera has been prepared and is being used to analyze requirements for idiotype-matching for cell collaboration and B cell receptor status during triggering. These experiments have demonstrated that pretreatment of T helper lymphocyte donors with anti-T15 serum leads to the generation of Lyt 1^-, 2, 3^+ suppressor cells which interfere with T-B lymphocyte collaboration in the antibody response to PC. Removal of the anti-idiotype induced suppressor T cells with anti-Lyb 2.1 serum reveals normal levels of T helper function, suggesting that suppression is at the level of T-B interaction or directed against the B cell. The elicitation of suppression requires both the original carrier and PC to be presented at the time of secondary challenge, suggesting that PC-specific B cells are not the primary target of suppression. It therefore appears that collaborative and suppressive T-B cell interactions are under complex regulatory control and that anti-idiotype antibodies can upset the T lymphocyte regulatory network.

All of these experiments are designed to gain a better insight into the molecular and cellular requirements for specific lymphocyte activation and to thus make possible a rational manipulation of immune responses in humans. These studies are informative for those wishing to immunize successfully newborn humans, for instance.

PUBLICATIONS:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00035-04 LI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Specificity in Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Dr. Vernon Alvarez, LCB/NCI
Dr. Joyce Schroer, LCI/NIAID
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Dr. William E. Paul, LI/NIAID
Dr. Garrett DuBois, LCB/LCI

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range objective of this project is to carefully re-examine the nature of binding specificity at antibody combining regions and other types of receptor sites. The effects of polyvalent binding on binding energy and specificity will be considered, and together these properties will be related to the behavior of immune systems. In particular, multispecific binding of disparate structures by individual antibody species is being studied through screening of specifically purified, radiolabeled antibodies with affinity chromatography columns. The ligands are bound to these columns using large haptenic reagents synthesized specially for this purpose using methods of peptide synthesis. Multispecific binding will be used for producing and isolating homogeneous antibody by cross-stimulation and immunoadsorption. The resulting preparations will be studied by amino acid sequencing in order to gain more insight into the nature of antibody diversity. Methods for improving sequence analysis are actively being pursued.

PHS-6040
(Rev. 10-76)

A number of chemical techniques and products that have been developed from the above work are being used in collaborative studies on molecular interpretation of cellular mechanisms in immune responses. Material employed include synthetic thymus-independent antigens.

Project Description

Objectives

The overall objective of the project is to gain more complete understanding of the molecular basis for specificity in immune responses. The binding properties of antibodies and other receptor-bearing molecules are to be studied and related to the behavior of immune systems. Specific objectives are the following:

1. To assess the generality and frequency of multispecific binding by individual antibody combining regions by means of screening experiments employing many diverse, structurally unrelated haptens.

2. To develop methodology required for such screening tests including the synthesis of numerous, large haptenic reagents.

3. To employ multispecific binding and cross-stimulation in raising high titers of highly restricted antibodies for primary structure analysis.

4. To develop improved methods for determining amino acid sequences, and to sequence variable regions of restricted, normally raised antibody light and heavy chains.

5. To relate findings from the above experiments to questions concerning the nature and extent of antibody diversity.

6. To help employ mentods and reagents developed in the above work in cooperative studies in immunological specificity at the cellular level.

Major Findings

During the past year I have made an extensive review of the literature on classical or solution-phase peptide synthesis. From the results of this rather time-consuming effort, I am writing a critique of peptide synthesis from the standpoint of minimal protection (chemical masking) of side chain functional groups which will be published in a forthcoming series on synthetic methods in peptide chemistry. This task is directly related to project objective 2, the synthesis of numerous, large haptenic reagents for use as specificity probes. The haptenic structures are being built in more or less systematic fashion as derivatives of small peptides. The tactics of minimal side chain protection serve well in reducing the labor involved in such syntheses. The written critique will be the first over-all review of peptide synthesis with minimal side chain blocking.

A number of primary intermediates for large haptens have been prepared which are based on dipeptides of glutamic acid (of the types, Glu-X and Glu ($-X^r$). These derivatives bear two suitable blocking groups and a potential attachment function such as methyl or ethyl esters of glycine, glycylglycine or β-alanine. The attachment arms are joined to the carboxyl groups of the varied amino acid components, X. Sixteen such peptide derivatives have been made; about half of the derivatives were crystallizable, and they were accordingly prepared in substantial quantity in a highly pure state. Eventually, the ester functions will be converted to hydrazides (for preparing reactive azides) and the other blocking groups will have been replaced with a great variety of terminal structures. The latter structures will bear many of the unmasked functional groups which are compatible with minimal protection tactics used in forming the peptide, or amide, bonds of the hapten. A similar group of peptide derivatives based on lysine in place of glutamic acid is currently being synthesized.

A collaborative study on improving the methodology of amino acid sequencing of peptides on solid phases has continued this past year with significant advances being achieved. This study has been carried out with Drs. E. Appella, G. DuBois and V. Alvarez (all of LCB/NCI). We have demonstrated that chemical derivatives of macroporous polystyrene can be prepared which are suitable for anchoring peptides to be sequenced by Edman degradations. Macroporous polystyrene particles are non-swelling and, like porous glass, do not cause blockage of reaction columns as do other supports. Unlike porous glass, however, our supports allow efficient and stable attachment of small peptides. The macroporous derivatives yield much cleaner degradation products than conventional supports (e.g., porous glass, low cross-linked polystyrene, and polyacrylics). This advantage should permit significantly higher sensitivity in the analysis of peptides. We have experimented with amino derivatives of the rigid polystyrene which possess a variety of spacing structures (polyethers, polyamines and polypeptides); most of these preparations have performed satisfactorily in automated solid-phase sequencing, and we are now evaluating their relative merit. Sequencing products from these resins (thiazolinones) have been successfully converted to phenylthiocarbamyl amino acid methylamides for identification and quantitation. Much of the above work has been reported (see publications).

During his visit in my laboratory last November, Dr. Hans Jörnvall (Karolinska Inst., Stockholm) completed a study with me on the use of methylaminolysis as a conversion reaction in manual Edman sequencing techniques. We were able to show the general usefullness of this reaction for preparing identifying derivatives in conjunction with the dansyl substractive procedures, especially where residues of glutamic acid, glutamine, aspartic acid, asparagine and tryptophan are encountered. These specific residues were formerly not identifiable after the usual acid digestions of dansyl peptides, and additional material and analytical procedures were required. The work will be reported soon in Analytical Biochemistry.

During the month of March, Drs. Aldo Previero and M.A. Coletti-Previero from Montpellier, France visited my laboratory and those of Drs. Appella (NCI) and Liu (FDA). During this time we were able to discuss a number of chemical problems related to amino acid sequencing. We initiated some

collaborative studies on the use of C-terminal oxazolinone derivatives of peptides for their covalent attachment to solid-phase supports and worked out some useful analytical techniques for following these reactions.

During the past year several collaborative studies were completed with B. Merchant, et al. (BOB/FDA) which dealt with the regulation of specific B-cell responses in defective CBA/N mice (see Publications under Merchant, Snippe, Lizzio, and Inman). CBA/N mice, possess an X-linked B-cell defect that renders them unresponsive to most thymic independent antigens. We demonstrated that these mice are unusually sensitive to blockade of their apparently normal PFC responses to haptens on thymic dependent antigens by conjugates of the same hapten on thymic independent (TI) carriers. As little as 10 nanograms of the latter given a few hours before immunization will virtually abolish the fourth day, direct PFC response to 50 µg of haptenated hemocyanin. TI carriers such as Ficoll and pneumococcal polysaccharide were hightly effective in such blockade when appropriately haptenated: It was shown that the haptenic groups must be covalently bound to the carrier, and that the blocking power increased with hapten density. Mice bearing B memory cells were refractory to such blockade, and the development of specific B-cell memory was not affected by prior blockade of the primary response. These findings suggest very different blockade susceptibilities for B cells or their precursors at various stages of differentiative development.

I participated in several other studies carried out in Dr. Merchant's laboratory which have been (or soon will be) reported in Immunology (see publications under Snippe, et al.): 1) Delayed type hypersensitivity (DH) in mice was provoked with different hapten-carrier conjugates mixed with the cationic, surface-active lipid, dimethyl dioctadecyl ammonium bromide. A broad array of cross-reactions involving small and enlarged haptens showed a relative lack of specificity in this kind of DH response. 2) A study was made of the pattern of delay in various aspects of humoral immune (PFC) responses in mice following cyclophosphamide treatment. A comparison of nude and nu/+ mice suggested that the presence of T cells has little influence on the recovery of B cells which are destined to become direct PFC. However, the recovery of B cells destined to produce indirect PFC responses is facilitated by the presence of T cells.

Finally, Dr. Joyce Schroer (LCI/NIAID) and I have developed a method of sensitizing erythrocytes with insulin by thiolation of red cell membranes followed by treatment with an insulin derivative bearing an SH-specific alkylating function (maleimide group). The insulin-bound red cells have given sensitive and reliable responses to anti-inulin antibodies in hemagglutination and PFC assays.

Significance to Biomedical Research and the Program of the Institute:

An essential characteristic of many immune mechanisms is the specificity of action which is directed by definitive chemical structures. An understanding of specificity in immune systems rests first upon an adequate knowledge of the scope and nature of specificity at the level of single receptor-determinant interactions. Therefore, a principal aim of this project is to carry out the first systematic and general exploration of the phenomen-

on of multispecific binding. Because of the diversity and availability of antibodies, these receptor-bearing molecules provide ideal models for such studies in addition to their being interesting as important components of many types of immune reactions.

Cooperative studies have proven the value of large haptenic reagents and other synthetic or semi-synthetic antigenic components in exploring specificity as it is manifested in more complex, multi-receptor-determinant interactions. Reagents or reactions developed for the explicit requirements of this project can have interesting applications in many basic studies in immunology such as those aimed at elucidating the mechanisms of B and T cell specific activation.

Future Course

Henceforth, the main effort of this project be directed torward studying multispecific binding by antibodies by affinity chromatography techniques. Considerable preliminary work has been completed, and I will be joined (Sept. 1, 1978) by a postdoctoral fellow whose principal concern will be to carry out this study with me.

Collaborative studies of sequencing methodology will be shifted more to Dr. Appella's laboratory where the experimental work will be done largely by Dr. Vernon Alvarez. Some chemical manipulations will be carried out in my lab by Dr. Alvarez.

Collaborative studies with Dr. Merchant (BOB/FDA) and Dr. Schroer (LCI/NIAID) will be continued.

Publications

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Jörnvall, H., Inman, J.K., and Appella, E.: Aminolysis of thiazolinones in manual amino acid sequence analysis. Direct detection of carboxyl, amide and tryptophan residues in conjunction with the dansyl-Edman method. Anal. Biochem. In press.

Honors

Recipient of NIH Director's Award, June 26, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00036-13 LI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)
Immunoglobulin Genetics: Regulation of Gene Expression and Lymphoid Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Rose G. Mage, Ph.D., Senior Investigator, LI/NIAID

Other Investigators: G.O. Young-Cooper, LI/NIAID
C.B. Alexander, LI/NIAID
R. Wilder, Research Associate, LI/NIAID

COOPERATING UNITS (if any) M.D. Cooper and A. Lawton, Univ. Alabama, Birmingham, Alabama; E. Appella, LCB/NCI; R. Asofsky, Chief, LMI/NIAID. In addition, we cooperate with a large number of investigators at NIH and elsewhere. Numerous investigators have received allotype-defined rabbits, reagents and allotyping information from our laboratory.

LAB/BRANCH
Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 1/2	2	3 1/2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Overall goals of this project are to: define the nature of the genes which code for, or otherwise affect the structure of immunoglobulins (Igs), to understand the regulatory processes which control Ig gene expression; to understand the mechanisms which lead to the synthesis of highly specific antibody molecules, and the nature of genes which influence and control immune responses. We prepare immunological reagents which detect structural differences between genetically controlled polymorphic forms of rabbit Igs (Ig allotypes) and between classes and subclasses, types and subtypes of their polypeptide chains. We have been using both immunological and chemical approaches to define the structural differences as well as to learn more about the chromosomal organization of genetic information for structure and expression of antibody molecules. We utilize the information and the reagents in studies including breeding experiments, investigation of the ontogeny and differentiation of cells which produce Igs intracellularly, as secreted products, and as membrane-associated receptors, and investigation of factors which influence and regulate phenotypic expression of alleles and chain types.

PHS-6040
(Rev. 10-76)

The objectives of this research program are to define the nature of the genes which code for, or otherwise affect the structure of immunoglobulins (Igs), to understand the regulatory processes which control Ig gene expression, to understand the mechanisms which lead to the synthesis of highly specific antibody molecules, and the nature of genes which influence and control immune responses.

For these studies, we prepare immunological reagents which detect structural differences between genetically controlled polymorphic forms of rabbit Igs (Ig allotypes) and between classes and subclasses, types and subtypes of their polypeptide chains. We have recently started to prepare reagents which may only react with cell surface membrane-associated Igs such as IgD or the postulated Ig-like receptor on T cells, as well as antibodies to other antigens which may be characteristic of cell surfaces of lymphoid cell populations. We use both immunological and chemical approaches to define structural differences and conduct genetic experiments in order to learn more about the chromosomal organization of genetic information related to Ig molecules.

New Methods

A number of methods have recently been developed or improved in this laboratory to aid in the conduct of these studies. For example, Sepharose CL-6B has been shown to be highly stable in 6M guanidine HCl and is suitable for molecular weight determinations in the range 3,000-80,000 daltons without presenting the problems of flow rates and low chemical and physical stability commonly encountered with other soft gels in 6M Gdn-HCl. This method was utilized in characterizing immunopeptides from the variable regions of heavy chains of limited heterogeneity and a3 allotype (Ansari and Mage, 1977, 1978, Ansari et al., 1978). In addition, a screening assay for rapid and quantitative measurements of anti-allotypes and allotypes in unknown sera was developed (Werblin and Mage, 1977). It has now been applied to cell-culture supernatants as well as serum samples. In addition, a modification of the labeling technique (reductive alkylation, by which tritium is introduced into proteins) has been developed which permits preparation of much more stable reagents which can be used for longer periods of time than previously used reagents labeled with ^{125}I (Wilder et al., manuscript in preparation). The method has also been used to radiolabel protein A. The protein A binding assay has been applied to radioimmunoassays, and to measurement and detection of cell surface receptors on lymphoid cells. We and others in the Laboratory of Immunology also plan to use this assay for the detection of antibodies produced by hybridoma cell lines.

Studies of Surface Igs

In the course of studies of the cell surface receptors of rabbit B and T cells, rabbit and mouse splenocytes were radioiodinated by the lactoperoxidase technique, extracted with non-ionic detergent, immunoprecipitated with high titered rabbit anti-kappa antisera, and compared by SDS-PAGE. Mouse sIg peaks were reproducibly larger in size than rabbit sIg peaks (often greater than 10 times). Neither differences in incorporation of label into the rabbit

cell surface, nor differences in average sIg density explain this result. Total TCA precipitable radioactivity was similar in each species. Estimation of the relative amounts of sIg in the mouse and rabbit by protein A binding assay, showed similar average sIg densities. Differences in detergent solubility, proteolytic lability, or antisera used also do not adequately account for this difference. The data support the concept that the lactoperoxidase radioiodination may fail to label or only poorly label some cell surface proteins or their subunits. Therefore caution is advised when interpreting relative peak sizes, as they may not reflect relative amounts of cell surface protein (Wilder, Yuen and Mage, submitted).

Based on previous biochemical studies of rabbit sIg, we postulated that if rabbits possessed an IgD-like molecule, its heavy chain might co-electrophorese with the μ heavy chain under reducing conditions on SDS-PAGE. Accordingly, we studied Ig from rabbit spleen cells radio-labeled biosynthetically or by cell surface iodination. Cell lysates were prepared and immuno-precipitation studies performed by standard techniques. Sequential absorptions in which total removal of μ allotype (n-locus) was followed by immunoprecipitation with anti-a (V_H) or anti-b (kappa) showed residual band(s) with relative mobility similar to standard μ chains on SDS-PAGE under reducing conditions. Similar immunoprecipitations with anti-gamma (γ) or anti-alpha (α) reagents failed to precipitate the residual band with μ mobility. The residual band was found in both radioiodinated and biosynthetically labeled lysates.

When direct immunoprecipitation was carried out in the absence of proteolytic enzyme inhibitors, the SDS-PAGE pattern of total Ig under reducing conditions frequently showed three peaks, corresponding to μ (MW∿70,000), light chain (MW ∿ 23,000) and an unidentified heavy chain (MW ∿ 65,000). Thus we have identified a "new Ig" in the rabbit that bears V_H and light chain, and lacks μ, γ and α allotypic markers. The molecule appears to be proteolytically labile, but has an undegraded heavy chain which co-electrophoreses with the μ chain on SDS-PAGE under reducing conditions. The similarities to published data on human and rat lymphocyte IgD, strongly suggest, but do not formally prove, that we have identified the rabbit homologue of IgD (Wilder, Yuen, Coyle and Mage, submitted).

A variety of approaches have been taken to identify cell surface receptors which possess Ig-like properties such as V_H region structures detectable with anti-allotype reagents. To date results in this laboratory have been largely negative, although experiments are still in progress. In a collaborative study with the laboratory of K. Rajewsky, Koln, Germany (Krawinkel et al., 1977), hapten binding receptors of sensitized lymphocytes in a nylon-wool enriched T-lymphocyte fraction were found to lack determinants of Ig constant domains but carry a (V_H) allotypic marker.

The approaches we have taken include examination of biosynthetically labeled or cell surface iodinated proteins or glycoprotein fractions by immunoprecipitation and SDS-PAGE, protein A binding assays, and immunofluorescence including analyses on the Fluorescence Activated Cell Sorter (with the collaboration of Dr. I. Scher). We have also attempted to develop

systems for stimulation of T cells in culture by altered-syngeneic, allo- or xeno-antigens in order to test for inhibitory effects of anti-a V_H allotype reagents.

So far we have found that cells and molecules with V_H a-allotypes also have determinants associated with the Ig of classical B cells (kappa allotypes or determinants detected with heteroantisera to constant regions of H and L chains). The same reagents used by Krawinkel et al. (1977) to obtain an "Ig-negative" fraction of hapten-binding receptors from rabbit cells have removed more than 90% of the a-allotype positive labeled Ig in biosynthetically labeled and concentrated culture supernatants. When the concentrated products of 5×10^8 thymocytes were analyzed in each immunoprecipitate, no Ig other than classical B cell products was detected. Similar results have been obtained with NP-40 extracts of cells. Cell sorter analyses of the cells of a2b5 and a3b4 homozygous rabbits using antibodies to specific (a1 anti-a2 and a1 anti-a3), control (a2 anti-a1, a3 anti-a1) and kappa allotypes (anti-b5 and anti-b4) detected Ig-positive and Ig-negative cell populations but failed to demonstrate a distinct population of a allotype positive cells which stained with different intensity from kappa positive cells or was present in detectable excess above the proportion of classical B cells detected in spleens and thymuses. We have now immunized chickens with $(Fab')_2$ fragments of rabbit IgG, and a goat with the isolated V_H portion of Ig-heavy chains and are analyzing the reactivities of these reagents with rabbit T and B cells. Further attempts to detect T cell Ig in plasma membrane preparations are also in progress.

Ontogeny of B Cells in Normal and Suppressed Rabbits

Pre-B cells in developing rabbits were identified by immunofluorescence as cell containing small amounts of cytoplasmic IgM (cIgM) but lacking surface immunoglobulin (sIg). During ontogeny, the first pre-B cells appeared in fetal liver at 23 days gestation, 2 days prior to the appearance of $sIgM^+$ B lymphocytes. Pre-B cells were relatively frequent in fetal and adult bone marrow, but were not found in other tissues except rarely in fetal spleen. Allelic exclusion is apparently established at this stage of development. At least in those individual pre-B and B lymphocytes from heterozygous rabbits producing detectable kappa chain allotypes, only one or the other alternative allele was present in amounts sufficient for detection. Development of isotype diversity among rabbit B lymphocytes followed the general pattern seen in mouse and man. Cells with sIgM were detected before birth. Expression of $sIgG^+$ was detected in neonatal rabbits on cells which were also $sIgM^+$ but in older animals most $sIgG^+$ cells lacked sIgM. Cells bearing sIgA were not found until 5-6 days of age, and had no other isotype detectable on their surface (Hayward et al., 1978 and submitted).

The effect of allotype suppression and subsequent neutralization on the expression of kappa light chain allotypes (b4 and b5) was examined in rabbit B and pre-B cells. Heterozygous b4b5 rabbits were injected with anti-b5 antibody at birth and 1-5 additional times during the first two weeks of life. The spleen, bone marrow and peripheral blood lymphocytes were examined at 14,

to or 23 days after birth. Some allotype-suppressed animals were neutralized by injection of b5 antigen 24 hours before sacrifice. Double immunofluorescent staining detected μ^+ B cells in the bone marrow which were also $b5^+$ in control heterozygotes whereas no $b5^+$ B cells were found in the marrow of rabbits suppressed for b5. In contrast, the ratio of $b4^+$ to $b5^+$ pre-B cells in the marrow was relatively constant in treated and untreated heterozygotes. Neutralization resulted in an increase in the proportion of B cells bearing the formerly suppressed allotype which was detectable 24 hours after neutralizing antigen was administered. The relative proportion of b allotypes expressed by pre-B cells is unaffected by allotype suppression and subsequent neutralization. However, chronic allotype suppression results in long-term depressed production of one allotype by B cells even in rabbits more than two years of age (Simons et al., presented at 12th International Leukocyte Culture Conference, and manuscript in preparation). Additional fluorescent antibody staining studies of PBL of suppressed and control heterozygotes are in progress. It appears that during the chronic stage of suppression, some rabbits recover a population of B cells which bear sIg of the suppressed allotype but relatively few secreting cells are produced. The relative proportions of b allotypes expressed by heterozygous rabbits are probably affected and influenced at more than one level of regulation.

Other Projects

The assay of Werblin and Mage (1977) as modified by Wilder et al. (see above) was used to complete analyses for anti-b5 in bleedings taken monthly over a 12-month period from b5-immunized b4 offspring of b4b5 mothers (and control animals). The maternally derived foreign b5 allotypic antigen has a tolerizing effect upon young rabbits immunized at two weeks and four weeks of age. Compared to matched controls, age of onset and magnitude of responses are affected (Popiel, Alexander, Young-Cooper, Werblin and Mage, in preparation).

The sequence of the V_L portion of a b4 light chain from anti-SIII antibody of restricted heterogeneity was completed. The amino-terminal sequence including the first hypervariable region is very similar to the sequences of six different light chains from rabbit antibodies to the Streptococcal A-variant carbohydrate studied by Braun and coworkers (Chersi, Mage and Appella, manuscript in preparation).

Breeding to detect recombination between genes in the heavy chain linkage groups has continued with no additional recombinants detected. A collaborative study which involved exchange of reagents and sera between this laboratory, those of Dray, Gilman-Sachs, Knight and Hanly, in Chicago, Wm. Mandy in Austin, Texas and R. Hamers in Brussels, Belgium, has resulted in better understanding and comparison of the μ-heavy chain allotypic determinants detected by the Chicago and Brussels groups, and re-evaluation of the heavy chain allotypes involved in the recombination events reported by Mage et al., Knight and Mandy, and Hamers et al. A cooperative backcross analysis for H locus allotypes is also in progress in collaboration with Dr. R.R. Fox, The

Jackson Laboratory, Bar Harbor, Me.

We have also started to breed rabbits with the BAS mutant trait obtained from A. Kelus, Basel Institute for Immunology, Basel, Switzerland. This mutant fails to produce kappa-type light chains.

A workshop-meeting on "Immunogenetics of the Rabbit" was held at the NIH under the sponsorship of NIAID on May 3-5, 1978. I chaired the conference which was organized with the help of Dr. T.J. Kindt, LIG, and Dr. Carl Cohen, IAIDP. The meeting brought together investigators working on various aspects of the immunogenetics of rabbits for exchange of information, increased cooperation, and delineation of future approaches to problems which may benefit from use of the unique attributes of this species. One of the achievements of the conference was that in addition to considerable exchange of information about Ig-allotypes, idiotypes and cellular immunology of rabbits, there was coverage of RLA, transplantation and biochemical polymorphisms. Numerous plans for future cooperation and exchange of information and reagents developed between individuals working in the various subject areas in North America, Europe and Japan.

Immunogenetics continues to be at the forefront of our understanding of the nature of structural and regulatory genes in complex mammalian systems. As knowledge of the chemistry, genetics and evolution of immunoglobulins increases it becomes one of the most valuable models for investigation and understanding of the functioning of complex genetic regions on chromosomes, and the molecular basis for cellular differentiation in higher organisms. The genetics, evolution and possibly the chemistry of cell surface isoantigens in the MHC appears analogous in many ways to the Ig systems. Studies of Ig allotypes have also provided models of direct relevance to evolution and immunogenetics of cell surface antigens. Understanding of the chemistry, genetics and evolution of Igs, the factors which control their genetic expression, and the differentiation pathways of the cells which produce them is thus of broad fundamental interest.

Most of the projects described are in progress and continuing. In connection with our continuing interest in rabbit IgD, we are attempting to raise alloantisera to rabbit IgD. We will also try to raise heteroantisera possibly via the hybridoma technique (see below). Anti-IgD reagents will be useful probes of functional subsets of rabbit B cells. As noted above, during chronic allotype suppression, cells with the formerly suppressed allotype appear in the peripheral blood when serum levels of the allotype are proportionally lower. We are planning to further evaluate the characteristics of the cells with suppressed allotype which reappear. We hope to use the FACS to determine whether such cells express sIgM only or both sIgD and sIgM. Reagents are also prepared and being evaluated for several collaborative studies which may eventually make use of FACS technology. With Dr. Asofsky and Ms Logan, we will be studying the expression of V_Ha markers and C_γ markers in doubly heterozygous rabbits such as a3,d11,e15/a2,d12,e14. With Drs. Cooper, Lawton, and Mr. Gathings, we will attempt to identify V_Ha allotypes

in pre-B cells and evaluate allelic exclusion or inclusion for heavy chains in these early B cell precursors. The BAS rabbit which fails to produce Ig with kappa type light chains will be studied to determine whether any pre-B cells contain kappa-positive Ig. Once the breeding colony of BAS rabbits is established, we plan further investigations of the molecular basis for the mutant phenotype. Preliminary evidence was presented at our Rabbit Immunogenetics meeting that the defect may be regulatory rather than genetic deletion of kappa structural genes. We are continuing our search for Ig-like material associated with the cell surface of rabbit T cells using anti-aV_H and additional hetero-antisera described above. We will be using both FACS and biochemical techniques in our continued studies. We are also planning to initiate antibody-producing hybridoma cell lines to obtain mouse antibodies to rabbit lymphoid cell surface antigens such as the rabbit equivalent of mouse Thy-1 antigen, sIgD, and other antigens.

Appendix - Contract Number NO1-AI-81406 Maintenance and Breeding of Rabbits of Known Genotype and their use for Immunological Studies (through 6/21/78) and NO1-AI-82565 6/22/78 and thereafter.

The objectives and methods are related to those outlined above. Rabbits and reagents produced and supplied through this contract were made available to numerous other investigators at the NIH and at laboratories throughout the world. The revised workscope of the contract which was re-advertised and competed this year reflects the research resources aspect of this contract more specifically than in the past. This contract has continued to provide outstanding support for production and maintenance of allotype-defined rabbits, immunization, bleeding and various short term and long term projects involving breeding, immunization and suppression.

Publications

Werblin, T. and Mage, R.: A screening assay for rapid and quantitative measurement of immunoglobulins and anti-immunoglobulins in unknown sera. J. Immunol. Methods. 16, 337-350, 1977.

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Mage, R.G.: Structure and expression of V_H and C_H allotypic determinants. In Mandel, T.E. (Ed.) Progress in Immunology III. Canberra, Australia, Aust. Ac. Sci. 1977. pp. 289-297.

Ansari, A.A. and Mage, R.G.: Molecular weight estimation of proteins using Sepharose CL-6B in guanidine hydrochloride. J. Chromat. 140: 98-102, 1977.

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Ansari, A.A. and Mage, R.: Immunochemical Studies of the a Allotypes of Rabbit Heavy Chain Variable Regions - I. Comparisons of a3 Allotypic Determinants on Normal IgG and IgG of Limited Heterogeneity by Radioimmunoassays with Purified Labeled Anti-Allotype Antibodies. Immunochem. 15: In press, 1978.

Ansari, A.A., Mage, R.G., Carta-Sorcini, M., Carta, S. and Appella, E.: Immunochemical Studies of Rabbit Heavy Chain Variable Regions - II. Immunological Properties of Peptides From Variable Regions of Heavy Chains of Limited Heterogeneity and a3 Allotype. Immunochem. 15: In press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00037-11 LI

PERIOD COVERED October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunogenetics of Mouse Immunoglobulins and Genetic Control of Antibody Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Rose Lieberman, Research Microbiologist, LI/NIAID

Other Investigators: Constantin Bona, LI/NIAID
Subbarao Bondada, LI/NIAID
Chau Chen Chien, LI/NIAID
William E. Paul, LI/NIAID
Herbert Morse, LMI/NIAID
Micheal Potter, LCBGY/NCI

COOPERATING UNITS (if any) Edward Boyse, Sloan Kettering Institute, N.Y.; Martin Weigert, Institute for Cancer Research, Fox Chase Center, Philadelphia, PA.

LAB/BRANCH Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Idiotypic determinants are unique antigenic determinants found on some or all antibodies of a given specificity but lacking from antibodies of a different specificity. In some instances, a substantial fraction of antibodies of a given specificity possess similar idiotypic determinants, which are referred to as cross reactive idiotypes (IdX's). This research program is mainly concerned with the characterization of IdX's expressed by anti-polysaccharide antibodies, most notably anti-bacterial levan (BL) and anti-inulin and the use of these systems to study the regulation of antibody expression. Our results indicated that an anti-IdX in the anti-BL system (E109) can directly suppress expression of B lymphocytes bearing that IdX and can indirectly suppress E109$^-$ anti-BL responses. The cellular and genetic factors regulating both direct and indirect suppression have been studied.

PHS-6040
(Rev. 10-76)

A. Immune Response to Levan

I. Kinetics and Ontogeny of Anti-Levan and Anti-Inulin Antibody Response and of Expression of Cross-Reactive Idiotype

The antibody response of young and adult BALB/c and of nu/nu BALB/C mice to bacterial levan (BL) was characterized for its specificity for levan and inulin and for the expression of a family of cross-reactive idiotypes (IdX) found on BALB/c inulin and levan binding myeloma proteins. Adult BALB/c immunized with BL develop high anti-levan and anti-inulin plaque forming cell (PFC) and hemagglutinin (HA) responses. Most of the anti-inulin antibodies exhibit the IdX. Response to BL is thymus independent as shown in nu/nu BALB/C mice but the titers of anti-inulin and -IdX are very low. 2-4 week old BALB/c mice develop anti-levan but not anti-inulin or IdX antibodies and mimic somewhat the low titers obtained in nu/nu mice suggesting that the thymus may influence the maturation of thymus independent lymphocytes. In addition, anti-IdX antibody secreting cells regularly appear in the course of the anti-BL response suggesting auto-anti-idiotype regulation of immune responses.

II. T-Independent Suppression of Anti-BL, Anti-Inulin and Anti-IdX (E109) Antibodies by Anti-IdX (E109) Antisera

Suppression of anti-inulin and anti-BL antibodies bearing the E109 IdX was induced in BALB/c mice with allo-anti-E109 IdX antiserum followed by BL immunization. Anti-inulin and anti-BL antibody suppression was short lived, whereas suppression of expression of E109 IdX persisted 30 days. Except for lower levels, the results in nu/nu BALB/c mice were essentially the same indicating that the immune response to BL and that the suppression of the IdX is T-cell independent. Similar determinations were done in C58, in Ig congenic B.C8, C.B20 and BAB 14 strains and in CXBG and CXBJ RI strains derived from BALB/c-C57BL crosses. The E109 component of the anti-BL response in strains which bear V_H genes derived from BALB/c (eg. C58, CXBG, CXBJ, BAB 14, and B.C8) can be suppressed by pretreatment with anti-E109. The anti-BL response of $E109^-$ strains (eg. C.B20 and C57BL) is not effected by anti-E109 treatment. This result indicates that direct suppression of the $E109^+$ component of the anti-BL response is a thymus-independent event which follows from the interaction of anti-E109 with E109-bearing molecules, probably on B cell membranes. Suppression of the $E109^-$ component of the anti-BL response (indirect suppression) is found in only some strains which possess structural genes for the E109 IdX (C58, CXBG, CXBJ and BAB 14). However, B.C8 mice, which are congenic to BALB/c at the $\underline{IgC_H}$ and $\underline{IgV_H}$ regions do not display indirect suppression indicating that genes outside the $\underline{IgC_H}$-region regulate indirect suppression.

III. Expression of Minor Clones Carrying Inulin IdX and IdI during Suppression of Dominant IdX in BALB/c nu/nu and Ig Congenic and Recombinant Strains Derived from BALB - C57BL Crosses

Anti-BL antibody of mice suppressed by treatment with anti-E109 were examined for IdX markers G, A and B which have been identified on BALB/c inulin and levan binding myeloma proteins and also for A48 IdI which is only found on A48 levan binding myeloma protein. G, B and A IdX and A48 IdI were found 5-10 days after BL immunization in normal BALB/c mice. In anti-E109 suppressed BALB/c challenged with BL, G, A and B idiotypes were suppressed for 30 days and G and B but not A continued to be suppressed at 60 days. However, suppressed nu/nu BALB/c mice expressed G and A48 IdI early and IdX A later. The expression of IdX A, B and G and A48 IdI varied considerably among the anti-E109 suppressed Ig congenic and recombinant strains.

IV. Response to Inulin (β2→1) and Grass Levan (β2→6) Polyfructosan

Anti-inulin ((β2→1) polyfructosan), anti-grass levan ((β2→6) polyfructosan) and IdX U61 PFC and hemagglutination inhibition (HI) responses were determined in BALB/c, C57BL and Ig congenic and recombinant mice derived from these 2 strains and in various other inbred strains immunized separately with grass levan and with inulin conjugated to Brucella abortus. BALB/c, C58, RIII, AL and A/He mice immunized with inulin BA developed 150 - 1000 anti-inulin PFC per 10^7 spleen cells, the majority of which were inhibitable by inulin and grass levan. 84-100% of BALB/c anti-inulin PFC carried the U61 IdX, whereas < 1-50% AL anti-inulin PFC exhibited U61 IdX. Inulin responses in C57BL mice were poor. Only 10-20% of these mice developed small numbers of anti-inulin PFC, none of which carried the U61 IdX. The B.C8 strain which is congenic to C57BL and has the BALB/c allotype gave a lower response than BALB/c while C.B20 mice, congenic to BALB/c but having the C57BL allotype, gave a higher response than C57BL. Thus, it appears that one gene controlling the immune response to inulin is linked to the V_H structural gene of BALB/c, and that other genes also influence the magnitude of the inulin immune response.

B. Preparation of Functionalized Derivatives of Inulin: Conjugation of Erythrocytes for Hemagglutination and Plaque-Forming Cell Assays

A method has been described for preparing derivatives of alkali-stable polysaccharides for coupling to sheep erythrocytes for use in HA and PFC assays or as immunogenic carriers. Inulin, a (β2→1) polyfructosan was partially derivatized with carboxyl amino-ethyl or butryl groups and coupled to SRBC following diazotization. The immunological reactivity of the inulin molecule was unaffected and the stability was markedly increased. It proved to be very efficient in biological testing such as HA and PFC.

C. Allotypes

A comprehensive review of mouse immunoglobulin allotypes was completed this past year including suggested nomenclature revision, methodology including immunization, preparation of alloantisera, and antibody assay methods, genetic markers of IgG2a, IgG, IgG1, IgA, IgM and IgD loci, λ_1L chain allotype, immune response genes, allotype suppression, latent allotypes, myeloma proteins producing more than one paraprotein, HC^1 complement genes, Ig congenic and recombinant strains, etc.

In addition, tables on immunoglobulin allotypes of the mouse were submitted for publication in the data book on Inbred and Genetically Defined Strains of Laboratory Animals published by FASEB.

D. Studies of Allotypes and Idiotypes of C.B20, NAB and Other Strains Congenic to BALB/c and having Different IgCH Genes

Plasmocytomas of a variety of classes have been induced in strains congenic to BALB/c but with different IgCH allotypes. Functionally similar myeloma proteins have been found among these. Homologous antisera to both the IgCH allotypes and the V_H-V_L idiotypes are being prepared to permit studies at the humoral and cellular levels of the role of the presentation of antigen in the immune response resulting in diversity of antibody.

E. λ_1L_1 allotype and λ_1^+ regulatory genetic markers are being determined in progeny of crosses of strains that are positive and negative for these markers, and may or may not carry the Lyt 1, Lyt 2, Lyt 5, PCA-1^+ and the Lyb 2.1 markers, to determine possible linkages among these markers. A variety of crosses carrying different combinations of these markers are presently being studied.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00038-06 LI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)
Structure and Activity Studies on Immunologically Important Cells and Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Dr. B. Tyran, LI/NIAID
Dr. L. Clement, LI/NIAID
Dr. E. Shevach, LI/NIAID
Ms. T. Basham, LI/NIAID
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USUHS

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	2.5	2

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Genes encoded in the I region control immune responses to many thymus-dependent antigens. The known products of these genes appear on cell surfaces as Ia antigens. The guinea pig Ia antigens are composed of α (33,000 m.w.) and β (25,000 m.w.) chains. The amino terminii of the β chains differ in amino acid sequence, but are homologous between loci within strain 13 and also between strains 13 and 2. Peptide mapping studies indicate that the guinea pig Ia α and β chains appear to have large regions of very similar structure.

Very little is known about the methods by which T cells control the immune response of B cells. In several systems it appears that soluble mediators are involved. In our studies on mitogen activation of lymphocytes we have found that stimulation of murine spleen and thymus cells with the pokeweed mitogen, Pa-2, causes T cells to produce a set (at least 7) of nonspecific (polyclonal) B cell stimulating or helper factors. Each of these factors causes an increase in the number of plaque forming cells in spleen cultures. At least one of these factors appears to act directly on the B cell and others require the presence of T cells for full activity.

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(Rev. 10-76)

Structure of the Ia Antigens.

Genes in the I-region of the major histocompatibility locus encode cell surface glycoproteins, Ia antigens, which seem to be a primary determinant of the regulation of the immune response. In guinea pigs, the better characterized Ia antigens are composed of two polypeptide chains, α (33,000 mw) and β (25,000 mw), which are linked by disulfide bonds in some cases and by non-covalent bonds in others. Using material from guinea pig spleen and lymph node cells metabolically labeled with ^{3}H-amino acids, we have confirmed and extended our previous studies on the partial amino acid sequence of the amino terminus of β chains. The data shows that this portion of the molecule varies between the different loci, but that the chains are homologous. Within the limits of this data, the homology between the disulfide bonded β chains from different strains (bearing the 4,5 marker in strain 2 and 7 in strain 13) is greater than between the 7 marker (bonded) and 3,5 (non-bonded) in strain 13.

To investigate the interior portions of the Ia glycoproteins, peptide maps were prepared by ion-exchange chromatography of the tryptic peptides from isolated radiolabeled chains. When the peptide maps of the α and β chains from the 3,5 locus were compared the preliminary data showed that many peptides elute at the same positions. This suggests that there may be a major conservation of structure between the two polypeptide chains of the Ia molecule.

These studies will be persued to determine the size and distribution of the conserved region(s) within the α and β chains and to locate the region(s) reflecting the genetic diversity of the Ia molecules.

Stimulation of Lymphocytes.

When added to murine spleen cell cultures, one of the pokeweed mitogens, Pa-2, appears to inhibit B cell mitoses and differentiation into immunoglobulin producing cells. At the same time Pa-2 stimulates mitoses in T cells. We have recently found that aliquots of the mitogen and cell free supernatants from these cultures stimulate immunoglobulin production (reverse plaque assay) in fresh spleen cell cultures. The B cell stimulating factor(s) (BSF) responsible for this activity is produced only during the first 24 hr period after Pa-2 stimulation. Blocking cell division does not abrogate BSF production. To determine the class of cell which was responsible for this activity, various cell populations were prepared by antibody dependent complement mediated cell lysis, FACS, and physical techniques. These enriched populations were then assayed for their abilities to produce BSF. The results clearly indicate that BSF is produced by T cells and not by B cells or macrophages.

To characterize this non-specific B cell helper factor, cell free supernatants from Pa-2 stimulated BALB/c spleen and thymus cultures were fractionated by gel permeation chromatography and affinity chromatography. The results show the presence of at least seven major components, each of which stimulates an increase in the number of plaque-forming cells when

assayed on spleen cells. At least one of these BSF's stimulates nude spleen cells and hence appears to act directly on the B cell. Other BSF's require the presence of T cells for maximum B cell stimulation. These factors also act as mitogens for the T cells and it is unclear whether they stimulate B cells in the presence of T cells or other T cell products, or if they stimulate T cells which in turn activate the B cell. In either case the final result of BSF addition is an increase in plaque forming cells. Preliminary experiments indicate that some, if not all, of these factors act across species lines, as well as across H-2 barriers.

Work is currently in progress to further delineate the biological activities of each of these factors, characterize their physical and chemical properties, and to determine the subclass of T cell responsible for their production.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00040-04

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Genetic Control of Immunocompetent Cell Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Ethan M. Shevach, Senior Investigator, LI/NIAID

Other Investigators: U. Yamashita, Visiting Fellow, LI/NIAID
R. Burger, Guest Worker, LI/NIAID
L. Clement, Research Associate, LI/NIAID
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COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range goal of this laboratory is to determine the function of the histocompatibility-linked immune response (Ir) genes in the regulation of the immune response and in the control of immunocompetent cell interactions. At present, the research effort is divided into two main areas: 1) the role of the I-region associated (Ia) antigens and Ir gene products in macrophage-T lymphocyte interaction, T lymphocyte-B lymphocyte interaction, and T lymphocyte antigen recognition. 2) Genetic, serological and physicochemical studies of the antigens of the guinea pig major histocompatibility complex.

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(Rev. 10-76)

I. Role of the I-Region in the Regulation of Immunocompetent Cell Interactions.

a. Macrophage-T Cell Interaction.

Our previous studies have concluded that the genetic restriction of the interaction of antigen-pulsed macrophages and primed T lymphocytes appears to be regulated entirely by the histocompatibility type of the macrophage used for initial sensitization, that T cells may be specifically sensitized to antigens associated with allogeneic macrophages, and that Ia antigen expression only by macrophages is sufficient for effective interaction with T cells. We have postulated that the site of expression of the histocompatibility-linked Ir genes may be at the level of the antigen presenting cell or macrophage rather than in the antigen-responsive T lymphocyte. Thus, macrophages from a non-responder animal would lack the I-region gene product necessary to process or present the relevant genetically controlled antigen. In order to test this hypothesis we have begun a detailed study of the biochemical basis of macrophage-lymphocyte interaction and we are attempting to gain an understanding at the molecular level of macrophage processing or presentation of antigen. We have used as a model antigen the simple trinitrophenyl (TNP) hapten which can easily be covalently coupled to the macrophage surface. We initially examined the effects of anti-TNP sera on the ability of TNP-modified macrophages to stimulate TNP-specific T cell proliferative responses in vitro. The addition of anti-TNP to TNP-modified macrophages immediately following conjugation inhibited their ability to stimulate TNP-specific T cell proliferation. This inhibition appeared to be specific for the TNP response since anti-TNP had no effect on the ability of TNP-modified macrophages pulsed with either tuberculin (PPD) or ovalbumin (OVA) to stimulate efficient PPD or OVA T cell responses. On the other hand, anti-TNP had no effect on the TNP-specific response to TNP-modified macrophages that had been cultured overnight before addition to primed T cells. These results suggest that the relevant TNP-determinants recognized by T cells are not exposed on the macrophage surface and raise the possibility that macrophages must process membrane conjugated TNP to create the immunogen recognized by T cells. In a parallel series of experiments we examined the effects of antisera directed against guinea pig Ia antigens on the ability of TNP-conjugated macrophages to stimulate TNP-specific T lymphocyte proliferation. Treatment of macrophages with anti-Ia sera for one hour before, one hour immediately after, or as late as 24 hours after TNP modification resulted in a reduced ability to stimulate the TNP-specific T cell. The inhibition produced by anti-Ia sera was specific and did not result from interference with the ability of macrophages to process TNP-conjugated membrane antigens in a non-specific manner. These results suggest that anti-Ia sera interfere with the development of a TNP-specific immunogen on the macrophage surface and strongly suggest that an association exists between TNP-modified membrane proteins and Ia antigens on the macrophage surface.

Two models for TNP conjugation to membrane proteins of the antigen presenting macrophage were considered. In the first model, the immunologically relevant TNP determinants are exclusively coupled to macrophage Ia antigens. In the second, macrophage Ia antigens are not conjugated by TNP-

modification, but a complex is formed between TNP-conjugated membrane antigens and unmodified Ia antigens. A biochemical analysis of TNP-modified macrophages demonstrated that guinea pig Ia antigens are not TNP-modified by treatment with TNP and TNP-modified Ia antigens do not comprise the immunogenic determinant recognized by the hapten-specific T cell. We therefore favor the model in which TNP-conjugated membrane proteins are complexed with Ia antigen on the surface of the macrophage. While we have not been able to prove the existence of this complex by the biochemical techniques employed, a complex maintained by the integrity of the intact cell membrane might very well exist.

b. T-Cell-B-Cell Interaction.

Although the histocompatibility requirements for the interaction of antigen-pulsed macrophages and the primed T cell which proliferates upon antigenic stimulation in vitro have been confirmed in several experimental systems, considerable controversy exists on the histocompatibility requirements for macrophage-T helper cell interaction and for T cell-B cell interaction. In order to better define the interaction of the macrophage with the T helper cell, carrier primed guinea pig T cells were positively selected in culture for 7 days with antigen-pulsed syngeneic or allogeneic macrophages. The selected cells were then mixed with hapten-primed B cells in a modified Mishell-Dutton assay. Only culture with syngeneic, but not allogeneic antigen-pulsed macrophages led to the selection of antigen-specific T helper cells and these selected T cells would only cooperate with syngeneic B cells. F_1 T cells selected with F_1 macrophages cooperated with B cells of both parents. However, when F_1 T cells were selected with antigen-pulsed parental macrophages, they would only collaborate with B cells of the same parental strain as the macrophages used in the selection culture. These results are strongly in support of the view that the primed T cell is activated by carrier determinants of the nominal antigen in association with Ia antigens on macrophages and the helper T cell, in turn, activates B cells which bear the same Ia antigens and determinants of the nominal antigen bound to immunoglobulin receptors on their surface. In addition, in experiments with antigens the response to which is controlled by I-linked genes, we demonstrated that primed (responder x non-responder)F_1 T cells would only collaborate with B cells of the responder parent. The defect appeared to be at the level of the B cell in that the addition to the cultures of antigen-presenting cells of the responder type did not restore the ability of F_1 T cells to collaborate with non-responder B cells.

C. Immunological Functions of Ia-Bearing Epidermal Langerhans Cells (LC).

In collaboration with Green (LI/NIAID) and Stingl and Katz (Dermatology Branch, NCI) we investigated the possible immunologic role of a morphologically well-characterized cell which accounts for about 3% of the cells in the mammalian epidermis. Previous studies had suggested that LC are related to cells of the monocyte-macrophage series in that they bear Fc-IgG receptors, C3 receptors and Ia antigens. Using inbred guinea pigs, we studied whether LC can mediate the same immunological functions as Ia bearing macrophages. Functional studies demonstrated that antigen-pulsed LC induced

a proliferative response in immune T cells which is comparable in magnitude to that seen with macrophages. In the mixed leukocyte reaction, LC were as effective stimulators as macrophages. Both the antigen-presenting and the MLR stimulatory capacities of LC enriched epidermal cells could be abrogated by pretreatment with anti-Ia sera and complement. The presence in the epidermis of Ia-bearing LC, capable of mediating the immunologic functions of Ia-bearing macrophages, has important clinical implications with regard to the role of LC as sensitizing cells in both contact hypersensitivity and skin graft rejection.

II. Genetic, Serologic and Physicochemical Studies of the I-region of the Guinea Pig MHC.

Our emphasis has shifted over the past year from studies directed to defining new specificities of the guinea pig I-region and to producing monospecific anti-Ia sera to the use of such sera in a variety of functional studies designed to probe the role of the Ia antigens in the regulation of the immune response. The results of some of these studies have been outlined above in the section on macrophage-T lymphocyte interaction. Other studies have included the development of techniques for the rapid purification of detergent solubilized Ia antigens by immunoabsorbent chromatography. The first step of this purification consisted of lentil lectin chromatography from which a glycoprotein-enriched fraction was obtained. The final step consisted of chromatography using Ia-specific immunoabsorbents, from which the antigens were eluted with glycine buffer. Antigen yields were determined by inhibition of cytotoxicity and mean yields were about 20%. Electrophoretic analysis demonstrated that a high degree of purification was obtained. This technique should offer a rapid and effective method for the isolation of Ia antigens.

In order to characterize the membrane proteins recognized by heterologous anti-lymphocyte sera (ALS) against a number of guinea pig lymphocyte populations, we performed immunoprecipitation experiments and polyacrylamide gel electrophoretic analysis. Suprisingly, although a number of different proteins were recognized by these sera, high-titered antibodies against guinea pig Ia antigens could be demonstrated in all ALS, including those produced against thymocytes. Selective absorptions and sequential precipitation experiments revealed significant levels of xenoantibodies that recognized specific Ia alloantigenic determinants. The presence of antibodies against Ia antigens in heterologous ALS may have important clinical implications as they may be an important factor in determining the immunosuppressive effects of such sera.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00147-03 LI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Mechanism of Activation of Thymus-Derived Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Ronald H. Schwartz, M.D., Ph.D., Sr. Investigator, LI/NIAID

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COOPERATING UNITS (if any) Immunology Branch, NCI; Metabolism Branch, NCI; Laboratory of Chemical Biology, NIAMDD; Department of Biochemistry and Molecular Biology, Northwestern University

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INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	4.5	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project deals with the function of immune response (Ir) genes in the activation of thymus-derived (T) lymphocytes. We have shown that antigen-presenting cells from low responder mice specifically fail to stimulate proliferation to the antigen under Ir gene control in primed (responder x non-responder)F_1 T lymphocytes, suggesting that one site of expression for Ir gene products is in the antigen-presenting cell. This cell type in the spleen bears Ia determinants, lacks immunoglobulin and Thy 1 determinants and is radio-resistant and adherent. The most likely candidate for this cell is one in the monocyte-macrophage lineage.

The T-cell proliferative response to defined protein antigens was investigated and for Staphylococcal nuclease and pigeon cytochrome c was found to be under the control of more than one Ir gene. In the case of pigeon cytochrome c, the antigenic determinant was localized to two amino acid substitutions at the C-terminal end of the molecule. These comprised a single antigenic site whose recognition was under the control of two complementing Ir-genes. Furthermore, a functional heteroclitic response was demonstrated for the first time at the T cell level.

In our studies of the role of Ir gene products in T-lymphocyte immune responses we have utilized the proliferative response of Peritoneal Exudate T-Lymphocyte Enriched cellS [PETLES], an assay developed in this laboratory several years ago. This immune response has been dissected into two components, antigen uptake and presentation by nonimmune cells and proliferation by primed T lymphocytes. During the past year we have explored the localization of Ir gene product expression and the characterization of the antigen-presenting cell.

Using the antigen, poly $(Glu^{60}Ala^{30}Tyr^{10})n$ [GAT], the immune response to which is under Ir gene control, we demonstrated that nonimmune high responder parental or F_1 spleen cells could present antigen well to F_1 responder PETLES primed to GAT, but that low responder parental spleen cells, in four different F_1 combinations, were unable to present GAT, although the low responder cells could present purified protein derivative of tuberculin (PPD). Mixtures of high and low responder spleen cells presented GAT as well as high responder cells alone. Furthermore, pretreatment of the spleen cells with anti-Thy 1 and complement or pretreatment of the PETLES with anti-Ly2.2 and complement failed to change these observations. These results indicate that the failure of low responder spleen cells to present GAT was not due to an active suppression mechanism occurring in the in vitro secondary cultures, and suggests instead that Ir gene products are expressed in antigen-presenting cells. Future experiments will be directed toward determining whether suppressor cells play any role during the initial priming of the T lymphocytes in vivo.

Because of the central role of the antigen-presenting cell in Ir gene controlled immune responses an effort was made to characterize this cell type in the splenic population. Through the use of cytotoxic elimination experiments and various adherent separation and radiation procedures it was determined that at least one antigen-presenting cell in the spleen cell population was an adherent, radioresistant, I-A and I-C bearing cell which lacked Ig and Thy 1 determinants. The most likely candidate for this cell type is one in the monocyte-macrophage lineage. However, the lack of presenting activity of cells from bone marrow and peritoneal exudates suggested that neither an immature promonocyte nor a highly activated thioglycollate-induced macrophage can serve this function. The presenting cell is probably some intermediate stage cell in the monocyte-macrophage series, possibly one developed enough to process antigen but not differentiated enough to destroy it completely.

In deciphering the mechanism of action of Ir gene products it was deemed necessary to investigate the types of antigenic determinants recognized by T lymphocytes to determine whether the repetoire of receptors on these cells was different from that of B lymphocyte receptors. To facilitate such experiments we turned to the globular proteins as antigens because they provided us with probes for which we possessed a thorough understanding of the amino acid sequence and a precise knowledge of their three-dimensional structure. Thus, we could localize the antigenic determinants and probe the specificity of the T cell receptor by cross-stimulation with evolutionary mutants and enzymatic and chemical fragments of the antigen. Initial studies were begun in collaboration with Drs. David Sachs, Jay Berzofsky, and

Alan Schechter on the genetic control of the T-lymphocyte proliferative response to Staphylococcal nuclease. Although the immune response to this antigen turned out to be very complex, we were able to show that the proliferative response to it was under Ir gene control. By using fragments of the molecule for restimulation in vitro we were able to demonstrate from the response patterns that several I region gene products were involved.

Because nuclease differed so much from any protein found in the mouse, its antigenic complexity proved too great to localize specific determinants. We therefore turned our attention to the cytochromes c, a family of proteins containing over 85 evolutionary variants, some of which differed from mouse cytochrome c by only a few amino acid substitutions. In conjunction with Drs. Michiel Ultee and Emanuel Margoliash at Northwestern University, we demonstrated that the T-lymphocyte proliferative response to pigeon cytochrome c in B10.A mice was under the control of two complementing MHC-linked Ir genes. Cross-stimulation of pigeon cytochrome primed PETLES with hippopotamus and Pekin duck cytochromes c enabled us to localize the antigenic site to two neighboring amino acid positions where pigeon and mouse cytochrome c differ; namely a glutamine for lysine substitution at position 100 and a lysine for glutamic acid substitution at position 104. Furthermore, we were able to demonstrate for the first time a functional heteroclitic response at the T cell level. Stimulation of pigeon cytochrome c primed PETLES with the cytochrome c from tobacco hornworm moth gave a larger proliferative response at a lower antigen concentration than the immunogen, pigeon cytochrome c. The difference between tobacco hornworm moth and pigeon cytochrome c in the antigenic site is that the former has the lysine residue at position 103 instead of 104. Thus, the B10.A T cell receptors probably have higher affinity for the glutamine and lysine side chains spaced by two amino acid residues rather than the three found in the pigeon molecule. Finally, mixing experiments demonstrated that most of the T cell clones were recognizing a single determinant involving both amino acid substitutions. This result showed for the first time that two Ir genes could control the immune response to a single antigenic determinant. Future experiments will be directed at determining whether the two gene control of this response requires both gene products to be expressed in the same antigen-presenting cell as we have demonstrated previously for the synthetic copolymer GLΦ.

Significance to Biomedical Research

Our attempts to understand the mechanism of T-lymphocyte activation are of fundamental importance to a basic understanding of how the immune system functions. Since this system plays a critical role in the bodies defense mechanisms against infectious diseases, tumors and transplanted tissues, achieving our goal would hopefully provide major insights into how to manipulate the immune system for the benefit of the patient.

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LABORATORY OF IMMUNOGENETICS
1978 Annual Report
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Annual Report of the Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, NIH, October 1, 1977 - September 30, 1978

Summary Report

The Laboratory of Immunogenetics conducts research aimed at elucidation of the genetic basis of immunologic function. A variety of immunologic techniques and structural chemical procedures are used to investigate the products of the genes encoding immunoglobulins and those encoding histocompatibility antigens. Earlier work concerning immunoglobulin genes which utilized the serologically detected allotypes and idiotypes of the rabbit antibodies and immunoglobulins has been extended in several instances by amino acid sequence analysis of the proteins involved. At present, we are additionally pursuing the possibility that the serologic markers of the rabbit immunoglobulins may mediate genetic control mechanisms and recently have begun studies on the histocompatibility antigens of the mouse and the rabbit. Radiochemical sequence analysis is being used to obtain the complete primary structure of a mouse transplantation antigen produced by tumor cells in culture. Because structural knowledge of rabbit histocompatibility antigens is at a rudimentary level studies are concerned with the basic nature of these antigens. A comparison of rabbit histocompatibility antigens to the more well characterized systems of the human and mouse will be carried out initially. Work in our laboratory also includes active investigation of new techniques that allow primary structural determination by nonradiochemical methods of molecules such as cell surface proteins that are available only in very small amounts. Currently, these investigations focus on the use of gas chromatography-mass spectrometry as a means of micro structure determination.

Research Accomplishments

Genetic Studies of Rabbit Immunoglobulins. A major effort of our genetic studies has concerned characterization of the allotype $b4^{v}$. It has been shown that this is a new allele of the group b allotypes, which are in the constant region of the rabbit kappa light chain. This allotype, which was first discovered in this laboratory during routine amino acid sequence analysis studies, has been shown by genetic analyses through eight generations of a rabbit family to be allelic to the other group b allotypes (Sogn and Kindt). Although it was initially observed that $b4^{v}$ had no serologic difference from b4, from which it differs by very few amino acid substitutions, recent studies using antisera absorbed on proteins from cottontail rabbits indicates that there are serologic differences between the two closely related light chains. These differences, however, require the presence of a heavy chain in order for the difference to be expressed (Smith, Sogn, Mandy and Kindt).

In studies on the idiotypes of homogeneous rabbit antibodies using antisera prepared by normal procedures, very little evidence of idiotype inheritance was observed. However, it was found that when the determinant measured by the antisera were limited in some fashion, for example, if the determinants of only the heavy or light chain were considered, inheritance and linkage could be observed (Yarmush and Sogn). More recently, an alternative method of limiting the specificity of idiotypic antisera was shown to

be possible. This involved manipulating the idiotypic reaction by hapten fractionation of the antisera (Mudgett, Coligan, and Kindt). A digalactosamine hapten was found to inhibit the binding of eight idiotypic antisera directed against different homogeneous anti-streptococcal group C polysaccharide antibodies. The sensitivity of the different reactions to inhibition by the same concentration of the hapten was found to be variable, ranging from 20 to 80% inhibition, suggesting that the idiotypic antisera contain two populations of antibodies. It was possible, using this hapten to fractionate the idiotypic antibodies into two populations with specificities for different regions of molecule.

Collaborative studies with Dr. Anne-Lise Børresen and Dr. Kare Berg at the University of Oslo on the high density lipoproteins of the rabbit have also been carried out. These studies are of interest to us because, in addition to the medical and biological significance of lipoprotein structure, their allotypes provide markers for two rabbit chromosomes unlinked to immunoglobulin or blood group substance genes. Progress has been made in structural studies of apoproteins implicated in HDL allotypic variation. Purification and characterization of the H1 1 antigen revealed a polypeptide with molecular weight of 20,000 by gel filtration. Amino acid analysis and amino terminal sequence of this protein were determined and gave indication that the polypeptide is unique.

Nonallelic Expression of Genes Encoding Rabbit Immunoglobulin. The notion that rabbit allotypes are inherited as autosomal codominant alleles has been challenged by observations of low concentrations of allotypes not detected by qualitative tests or predicted by parental genotypes. In the past year, molecules with so-called "latent" allotypes have been isolated from the sera of pedigreed rabbits and shown to be indistinguishable from normal allotypes. Most trivial explanations for these observations have been eliminated in recent experiments. Current research focuses on attempts to describe the role of the allotypes in control of immunoglobulin genes and on methods to provoke expression of latent allotypes in vitro and in vivo with various mitogens and other pharmacologic agents (Yarmush and Kindt).

Structural Studies of Rabbit Immunoglobulins. In several instances we have been able to correlate serologic findings with primary structure of the molecules involved. Such studies also serve to locate the antigenic determinants followed in our serologic studies. We have used as our model system homogeneous rabbit antibodies directed against streptococcal carbohydrate from the Group C streptococcus. In the past year, the partial amino acid sequence was determined for two rabbit light chains with allotype b9 from homogeneous antistreptococcal antibodies. These antibodies were isolated from the same rabbit and shared idiotypic determinants. The first hypervariable regions of the two antibodies were identical giving a basis for the shared idiotypy. It was further found that the difference between the variable region amino acid sequence of light chains with allotype b9 differs very little from that of allotype b4, whereas the constant regions of these light chains may differ by as much as 33% (Fraser and Kindt).

The allotype $b4^{V}$ mentioned above was discovered when constant region sequence studies were carried out on a b4 light chain. The sequence of this

constant region is known for 60 residues, approximately 60 more remain to be determined. Studies on this light chain continue. Thus far, only two differences between b4 and b4^{0} have been revealed. These are at positions 121 and 124 (Sogn).

The partial amino acid sequence of a rabbit H chain which lacks all group a markers has been determined and reported. Comparison of the sequence of this heavy chain with that of the human heavy chain of subgroup III shows 78% homology, suggesting that a common ancestral variable region gene existed in mammals prior to speciation. In contrast to the differences between the variable regions of the a-positive and a-negative H chains, their constant regions are structurally identical. This finding provides formal support for the concept that two genes can encode one immunoglobulin polypeptide chain and furthermore demonstrate the existence of variable region subgroups in the rabbit (Johnstone and Kindt).

Precursors to Immunoglobulin Molecules. When the messenger RNA molecules for immunoglobulins are translated in in vitro systems, products are produced which are larger than those found in vivo. It has been found that the amino termini of the precursor protein are extended by as many as 30 amino acids when compared to their mature counterparts. In collaboration with Alan Williamson and Helen Singer at the University of Glasgow, we have carried out radiochemical amino acid sequence analysis on precursors of the heavy and light chain of an immunoglobulin produced by a plasmacytoma induced in a mouse of the C3H strain. Preliminary results indicated distinct lack of homology to the three previously reported light chain precursors and to the one previously determined heavy chain precursor. All of the reported sequences are for molecules isolated from tumors derived from the BALB/c mouse strain. Obviously, a complete description of genes encoding immunoglobulin chains will require precise definition of the precursor gene product. It may be questioned, for example, whether a separate precursor gene exists for each variable region gene, or if a single precursor serves for one subgroup or one L chain type (Gates).

Structural Analysis of Murine Transplantation Antigens. Newly developed radiochemical amino acid sequence techniques have allowed sequence analysis of the glycoproteins specified by the murine H-2K^{b} gene. This work has been carried out in collaboration with Dr. Stanley Nathenson of Albert Einstein University. Thus far, all amino acids, with the exception of aspartic acid and asparagine, have been incorporated into products produced by the EL4 tumor line. The product, H-2.33 is isolated from this tumor line by immunoprecipitation from detergent lysed cells. Progress toward complete amino acid sequence of the H-2K^{b} glycoprotein includes 151 assignments in 170 positions to date. Comparisons of our sequence data to the limited amount of information available for other murine MHC products indicate homologies ranging around 85% for the other H-2K and H-2D products for which information is available. When our information is compared to that available for the human HLA B7 and A2 molecules for which approximately 124 positions are available for comparison, a homology of about 73% is observed. No significant homology to immunoglobulins has been noted (Coligan).

Rabbit Histocompatibility Antigens. Preliminary studies have involved isolation and purification of histocompatibility antigens of the rabbit. Current work has concentrated on one of the eight serologically defined haplotypes in the domestic rabbit. Sources of antigens for this work have been peripheral blood lymphocytes and spleen cells from Bar Harbor B strain rabbits. Antigen isolation has been carried out by detergent solubilization of cell membranes from cells that were intrinsically radiolabeled by growth in cultures containing tritiated amino acids. The glycoprotein fraction isolated and analyzed by SDS polyacrylamide gel electrophoresis indicates there are two major classes of antigens: one with a molecular weight of 43,000 which has associated with it a B_2-microglobulin subunit. A second antigen has an apparent molecular weight of 32,000. A fraction with a molecular weight of 43,000 has been isolated by affinity chromatography and preliminary sequence analysis reveal homology to human and mouse histocompatibility antigens. Structural studies on these antigens and also on B_2-microglobulin from the rabbit are continuing (Kimball, Gates, Coligan and Kindt).

Mass Spectrometric Sequence Analysis of Polypeptides. A recent line of work is aimed at development of rapid, sensitive methods for polypeptide sequence analysis and application of these methods to molecules under study in our laboratory. A particularly promising approach involves mass spectrometric methods of amino acid sequence analysis. Two major methods are under investigation. One involves hydrolysis of large peptides into dipeptides, derivatization of these with trimethylsilyl reagent, and analysis of the resulting peptides by GC-MS. Another involves derivatization of smaller peptides and introduction of these by the direct probe method into the mass spectrometer. Recently, the sensitivity of the former method was improved by the use of chemical ionization (CI) mass spectrometry. The CI mode was found to be superior to the earlier used electron impact mode for dipeptide identification because intensity of the molecular weight determining ions was much greater (Krutzsch). Studies continue on computer aided analysis of the data obtained in these studies (Fraser and Krutzsch).

The proteins chosen to test the mass spectrometric method are heavy chains from rabbit antibody molecules. These molecules are especially useful because the rabbit heavy chains are not amenable to routine sequence analysis technique. Also, they have large sequence regions that are constant and therefore useful for controls on the new methodology (Krutzsch, Kindt and Fraser).

Carbohydrate and Glycoprotein Antigens of Microbial Cell Walls. Personnel currently working in LIG have in the past several years determined structures for several polysaccharides of interest to our studies. The availability of expertise and equipment for carbohydrate structure analysis has led to the initiation of several collaborative projects concerning immunologically interesting polysaccharides. These include the polysaccharides from the following pathogenic organisms: Group B Streptococcus, Staphylococcus aureus, and Schistosoma mansonii. It is an objective of our studies to determine complete structures for these polysaccharides.

The most developed area involves the group-specific polysaccharide of the Group B Streptococcus, which is isolated from the cell walls of this organism. Composition of this antigen is 50% rhamnose, 10% galactose, 10% Nacetylglucosamine. In addition, the analysis of the carbohydrate shows that a previously unidentified carbohydrate is present in its structure. Although considerable data have been collected concerning this unknown carbohydrate, its true identity remains obscure (Coligan).

Other studies currently underway include analysis of a polysaccharide isolated from the eggs of S. mansonii. This molecule, which may be a substituted dextran, will be studied by carbohydrate structural techniques involving gas chromatographic and mass spectrometric analysis. In addition, mass spectrometric methods will be used to study a lipoteichoic acid from S. aureus. Data thus far reveal that D-alanine is the only amino acid in the preparation and that there are several fatty acids in the structure (Fraser).

Honors and Awards

Dr. Kindt continues to serve on the editorial boards of the Journal of Immunology, Proceedings of the Society for Experimental Biology and Medicine and the Zeitschrift fur Immunitatsforschung, and has recently been elected to serve on the editorial boards of the Journal of Experimental Medicine and Immunochemistry. Dr. Blair Fraser was invited to serve on the editorial board of the Journal of Immunology. Dr. Kindt was asked to present data at a meeting on the streptococcus which was held in Trinidad and was also invited to participate in a symposium at the meeting of the American Society for Microbiology. Dr. Coligan was invited to present his data on the primary structure of murine transplantation antigens at a symposium at the FASEB meeting in Atlanta and Dr. John Sogn served as chairman for a session on Immunogenetics at the FASEB meeting. Drs. Kindt, Coligan and Sogn were invited by the local chapter of the Society for Experimental Biology and Medicine to present an overview of the work of the laboratory. Dr. Kindt was appointed to the program committee of the AAI and has been reappointed adjunct associate professor at The Rockefeller University in New York.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00166-01 LIG

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Chemical Characterization of Rabbit Histocompatibility Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Thomas J. Kindt	Chief, LIG	LIG	NIAID
Others:	Dr. Edward S. Kimball	Staff Fellow	LIG	NIAID
	Dr. John E. Coligan	Research Microbiologist	LIG	NIAID
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This laboratory has been engaged in the isolation and purification of histocompatibility antigens of the rabbit. There are at least eight serologically defined haplotypes in the domestic rabbit. Current work has concentrated on the RL-A^c haplotype. The major sources of antigen have been peripheral blood lymphocytes from Bar Harbor B-strain rabbits and a tumor cell line derived from this inbred rabbit strain. Antigen isolation has been carried out by detergent solubilization of cell membranes that were intrinsically labelled with tritiated amino acids. Glycoproteins have been isolated and analyzed by SDS-polyacrylamide gel electrophoresis indicating that there are two major classes of antigens. The first has a molecular weight of 43,000 and has associated with it a beta-2 microglobulin (B_2m) sub-unit. The second antigen type has an apparent molecular weight of 32,000. Structural studies of rabbit B_2m are currently underway.

PHS-6040
(Rev. 10-76)

Chemical Characterization of Rabbit Histocompatibility Antigens

Graft rejection and control of the ability to manufacture appropriate immunoglobulin molecules are directed by products of genes residing in the major histocompatibility complex. Little is known about the structure and function of the major histocompatibility gene products although preliminary work has been reported for proteins from the mouse and human. It is our intention to use the rabbit as a third species so as to be able to compare MHC gene products structures. There are at least eight serologically defined haplotypes in the domestic rabbit.

Current work has concentrated on the RLA^c haplotype. The major sources of antigen have been peripheral blood lymphocytes from Bar Harbor B strain rabbits and the tumor cell line derived from this inbred rabbit strain. Antigen isolation has been carried out by detergent solubilization of cell membranes that were intrinsically labeled with tritiated amino acids. This is followed by affinity chromatography to obtain a more purified fraction.

Glycoproteins have been isolated and analyzed by SDS polyacrylamide gel electrophoresis indicating that there are two major classes of antigens. The first has a molecular weight of 43,000 and has associated with it a B_2-microglobulin (B_2m) subunit. The second antigen type has an apparent molecular weight of 32,000. The 43,000 molecular weight component has been isolated from the glycoprotein pool by antibody affinity column chromatography using a sheep anti-rabbit B_2m antibody. H-2 and HLA antigens have been shown to be noncovalently associated with B_2m. By analogy, therefore, the 43,000 dalton protein is in all likelihood the RLA^c molecule. Studies to discern its identity by immunological methods and by protein sequence analysis are currently underway. The 32,000 dalton protein is not associated with B_2m. It nevertheless constitutes a major proportion of the glycoprotein pool and has been isolated from the unbound fraction of the anti-B_2m column by ion exchange chromatography. This protein is being subjected to extensive immunological and structural analyses.

In addition, we are currently engaged in establishing the complete amino acid sequence of rabbit B_2m.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00167-01 LIG

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mass Spectrometry and Sequence Analysis of Polypeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Henry C. Krutzsch	Sr. Staff Fellow	LIG NIAID
Others:	Dr. Thomas J. Kindt	Chief, LIG	LIG NIAID
	Dr. Blair A. Fraser	Staff Fellow	LIG NIAID

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A major need in immunogenetic studies are fast, reproducible and sensitive methods for amino acid sequence analysis of proteins. Mass spectrometric (MS) techniques provide a promising means of meeting these goals. One method utilizing MS presently under study involves enzymatic digestion of polypeptides into dipeptides using Dipeptidyl Aminopeptidase (DAP method). Another employs direct introduction (DI) of derivatized peptides for analysis. Computer assisted data analysis programs for both MS methods are also under development.

The DAP and DI methods are being tested using rabbit heavy chains as model proteins because these proteins are not readily amenable to routine sequence analysis techniques. A further advantage of H chains is that they have regions of known constant sequences that are useful as controls on the new methodology.

PHS-6040
(Rev. 10-76)

Mass Spectrometry and Sequence Analysis of Polypeptides

It is the aim of this program to develop rapid and sensitive methods for polypeptide sequence analysis and to apply these methods to molecules of immunogenetic interest. Development of this method will include devising strategies for isolation and purification of peptides in amounts sufficient for characterization. These techniques will facilitate polypeptide sequence analysis, helping to determine more rapidly the primary structures of immunogenetically important proteins such as antibodies and histocompatibility antigens.

An LKB Model 2091 EI-CI mass spectrometer was used to determine the chemical ionization (CI) mass spectral characteristics of trimethylsilylated (Me_3Si) dipeptides. Samples were usually introduced into the mass spectrometer via gas chromatography. The CI mass spectral characteristics of Me_3Si dipeptides then allow identification of dipeptides resulting from DAP digestion of polypeptides. Alignment of these dipeptides into the sequence of the polypeptide is achieved using the dipeptides from a second DAP digestion of the polypeptide after it has been modified by a single Edman step.

Some new aspects of the DAP/GC-MS method for polypeptide sequence analysis were investigated. The chemical ionization (CI) mass spectral characteristics of a number (40) of Me_3Si dipeptides containing all 20 common amino acids at the amino and carboxyl positions were determined and compared to their electron impact (EI) mass spectra. The CI mass spectra typically contain three significant ions of high abundance that are used for dipeptide identification: the ion from the N-terminal residue following -cleavage of the central CH-CO bond, $(Me_3Si\text{-}NH{=}CHR)^+$, which yields the sequence of the dipeptide, and the ion resulting from loss of a silyl methyl group $(M\text{-}CH_3)^+$ and the protonated molecular ion $(M + 1)^+$ which yield the dipeptide molecular weight. The pattern of fragmentations established in these investigations should allow identification of any unknown Me_3Si dipeptide. The CI mode was found to be superior in sensitivity to the EI mode for dipeptide identification because the intensity of the molecular weight determining ions relative to the sequence determining (-cleavage) ion is greater in the CI mode, while the obtainable intensity of the -cleavage ion is similar in both modes.

The protein presently being studies is the N-terminal 225 residue fragment resulting from CNBr cleavage of a rabbit (#4135, a3 allotype) homogeneous antibody heavy chain. The general protocol used for sequence analysis is as follows: 1) complete reduction and alkylation; 2) lysine blockade with citraconic anhydride and limited tryptic proteolysis; 3) partial sequence analysis and further tryptic digestion; 4) generation of overlapping peptides with other enzymes such as chymotrypsin.

Publications

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Aminopeptidase Method of Polypeptide Sequence Analysis. In Hirs, C.H.W. and Timasheff, S.N. (Eds.): Methods in Enzymology. Academic Press, New York, 1977, pp. 391-404.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00168-01 LIG

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Structure of Rabbit Immunoglobulin and Antibody Heavy and Light Chains

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Others:	Dr. John A. Sogn	Sr. Staff Fellow	LIG	NIAID
	Dr. Henry Krutzsch	Sr. Staff Fellow	LIG	NIAID
	Dr. Blair A. Fraser	Staff Fellow	LIG	NIAID

COOPERATING UNITS (if any)

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Laboratory of Immunogenetics

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Immunogenetics Research Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Homogeneous rabbit antibodies to streptococcal carbohydrates, are used to investigate structural aspects of the allotypic and idiotypic markers of immunoglobulins. The partial amino acid sequence was determined for two rabbit light chains with allotype b9 from two homogeneous anti-streptococcal antibodies displaying shared idiotypy. Structural work on b4 light chains continues.

The partial amino acid sequence of rabbit a-negative heavy chain was determined and reported. In addition, three rabbit heavy chains from three homogeneous antibodies, two chains being allotype a3 and one chain being allotype a1, are presently under investigation.

Studies such as these that combine serological and structural investigation of antibodies have the potential to define genetic mechanisms involved in antibody synthesis.

PHS-6040
(Rev. 10-76)

Structure of Rabbit Immunoglobulin and Antibody Heavy and Light Chains

The structural aspects of serologically detected polymorphic forms of rabbit immunoglobulins will aid in understanding the genetic mechanisms involved in antibody synthesis. Study of the covalent structure of immunoglobulins involves both separation of the protein into component peptides and amino acid sequence analysis of the component peptides. Separation of heavy and light chains from rabbit IgG is a standard procedure but structural analysis of the components must be treated in a unique manner depending on the chain and its allotypic specificities. During this past year, we have made progress in determining structural features of light and heavy chains representing various allotypes.

In the area of light chain structure, the amino acid sequence was determined for residues 1 to 88 and residues 110 to 147 for a rabbit light chain with allotype b9 from a homogeneous anti-streptococcal antibody. The amino acid sequence of the light chain from a second antibody of identical allotype was partially determined.

In spite of large structural differences in constant region sequence between b4 and b9 light chains, the variable regions of the b9 antibodies are quite similar to those reported for b4 light chains. Both chains bear a b9-specific substitution (glutamic acid) at position 16. One chain also had substitutions at positions 70 and 81 that may be exclusive to variable regions of light chains with the b9 allotype. These allotype associated V_1 differences offer support to the notion that structural genes for the C_1 region are either linked to distinct V_1 gene complexes or that certain variable regions are expressed only in concert with certain C_1 regions.

Structural studies are continuing on light chains of other allotypes. More specifically, the newly discovered $b4^v$ allotype is being structurally characterized. In doing this work, new methods of chain cleavage and separation are being developed to overcome previous impasses to rapid analysis of light chain structure.

In the area of heavy chain structure, the partial amino acid sequence of a rabbit a-negative heavy chain has been determined for residues 1-43 and for residues 64-120. Comparison of the sequence with the human heavy chain subgroup III shows 78% homology suggesting that a common ancestral variable region gene existed in mammals prior to speciation. The constant region of the a-negative chain is structurally identical to that of a-positive chains whereas the variable region differs substantially between a-positive and a-negative molecules. These findings support the concept that two genes encode one immunoglobulin polypeptide chain and demonstrate the existence of variable region subgroups in the rabbit. A novel approach to the initial fragmentation of the heavy chain was developed in this study. This method, which involved digestion of the intact chain with V8 protease, produced a free N-terminus and should have wide application in future studies on heavy chains with blocked amino terminals.

Structural studies are under way on three other heavy chains from homogeneous rabbit antibodies. These heavy chains represent the a1 and a3 allotypes and will serve as prototypes for future structural work. New methods of peptide isolation and detection have been developed and are currently being applied to obtain peptides from these chains to allow complete structural analysis.

In addition to work on rabbit immunoglobulins, amino acid sequence analysis of the apoA-I of rabbit high density lipoprotein and the R-67 antigen of rabbit high density lipoprotein have been reported.

Publications

Børresen, A.L. and Kindt, T.J. Purification and partial characterization of the APO A-1 of rabbit high density lipoprotein. J. Immunogenetics 5: 5-12, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00169-01 LIG

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Primary Structural Analysis of Murine Transplantation Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. John E. Coligan	Research Microbiologist	LIG NIAID
Others:	Dr. Thomas J. Kindt	Chief, LIG	LIG NIAID
	Frederick T. Gates	Staff Fellow	LIG NIAID

COOPERATING UNITS (if any)
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genes in the mouse H-2 major histocompatibility complex determine a diversity of functions associated with immune recognition and reaction. Two of these genes, H-2K and H-2D, encode the major antigens involved in allograft rejection; a feature undoubtedly related to the genetic polymorphism of these H-2 molecules. In addition, these molecules play a role in T-lymphocyte responses to altered cell-surface antigens. These H-2 molecules are integral membrane glycoproteins and are associated on the cell surface with B_2-microglobulin. The purpose of this work is to determine the primary structure of these molecules in order that we may better understand their function and mechanism of action. Toward this goal, approximately 40% of the primary structure of the $H\text{-}2K^b$ molecule has been elucidated. Structural studies on $H\text{-}2D^b$ and murine B_2-microglobulin have been initiated.

Primary Structural Analysis of Murine Transplantation Antigens

In order to relate structural differences among mouse H-2 glycoproteins to changes in their biological activity and to examine various genetic and evolutionary relationships among the genes of the major histocompatibility complex, the complete amino acid sequences of the H-2K and H-2D glycoproteins are being determined.

Because of the limited amounts of these materials available, radiochemical methodology has been developed in order to pursue these studies. H-2 molecules are intrinsically radiolabeled by growing appropriate tumor cell lines in the presence of radioactive amino acids. Initial studies have utilized the EL4.BU ($H\text{-}2^b$) lymphoblastoid cell line. Following detergent extraction, the H-2 glycoproteins are isolated by immunoprecipitation with alloantiserum. Peptide fragments are produced by CNBr cleavage at methionine residues and are isolated by gel filtration and ion exchange chromatographic procedures.

Newly developed, radiochemical amino acid sequencing techniques have allowed amino acid sequence analysis of five major CNBr fragments of the glycoprotein specified by the murine $H\text{-}2K^b$ gene. All amino acids except aspartic acid and asparagine have been assigned in 153 of 170 possible positions in the NH_2-terminal portions of each of these fragments. These assignments represent approximately 50% of the total sequence of these fragments. These fragments have been aligned and represent the NH_2-terminal 80% of the intact molecule.

The NH_2-terminal sequence obtained for the $H\text{-}2K^b$ molecule was compared to the limited sequence information available for other MHC gene products. An 84% homology (16 of 19 residues) to the $H\text{-}2K^q$ and $H\text{-}2K^k$ molecules, which are identical to one another in the positions compared, was observed. Comparison of our data with human HLA-A2 and B7 molecules reveals 73% homology for the 124 positions presently available for comparison.

A major goal of our structural studies on the $H\text{-}2K^b$ glycoprotein is to locate positions of structural changes in the H-2K molecules isolated from the series of $H\text{-}2K^b$ mutants which have been identified. Strains of the $H\text{-}2K^b$ mutant series show strong histogenic reactivity *in vivo* and MLR and CML *in vitro* with the parents. In five of the mutants that have been examined, there appear to be only one or two amino acid interchanges between the H-2K molecules, produced by the parent and mutant strain. Further studies on the precise nature of the amino acid interchanges in these and other H-2K molecules of this mutant series will serve to localize the region of the molecule in which polymorphism can serve as a specific and effective signal for recognition by the immune system.

An added impetus to the study of these molecules is given by the relationships in humans between the homologous loci (HLA A and B) and predisposition to certain diseases. Furthermore, molecules coded for by

the K and D loci of the major histocompatibility complex appear to play a major role in the stimulation and specificity of T-lymphocyte response to virally induced and other cell surface antigens.

Currently, smaller peptides are being generated from the CNBr fragments of the H-2K^{b} molecule in order that the complete amino acid sequence may be determined. In addition, structural studies have been initiated on the H-2D^{b}, H-2K^{d} and H-2D^{d} molecules.

Publications

Ewenstein, B.M., Nisizawa, T., Uehara, H., Nathenson, S.G., Coligan, J.E., and Kindt, T.J. Primary structure of murine MHC alloantigens: I. Isolation, biochemical characterization and preliminary alignment of CNBr fragments from the H-2K^{b} glycoprotein. Proc. Natl. Acad. of Sciences, USA 75: 2909-2913, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00170-01 LIG

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Structural Studies on Precursors to Immunoglobulin Molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Thomas J. Kindt	Chief, LIG	LIG	NIAID
Others:	Dr. Frederick T. Gates, III	Staff Fellow	LIG	NIAID

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The in vitro translation of the mRNA for some proteins yields products which are larger than those found in vivo. The amino termini of these precursor proteins are extended by up to thirty amino acids when compared to their mature counterparts. Precursor sequences have been reported for murine (plasmacytoma) immunoglobulin light and heavy chains, with the conclusion that these sequences represent extensions of the variable region genes. In order to better understand the multi-gene nature of immunoglobulin genes, the purpose of this work is 1) to sequence other mouse immunoglobulin precursor molecules for comparison and 2) to establish whether such precursors occur in the rabbit. This requires the isolation and in vitro translation of immunoglobulin mRNA, followed by radiosequence analysis of the radioactive product.

PHS-6040
(Rev. 10-76)

Structural Studies on Precursors to Immunoglobulin Molecules

Peptides of as many as thirty amino acids may be present at the amino terminus of a protein when it is synthesized and these peptides may then be cleaved from the protein, presumably during the secretion process. These precursor peptides, which are hydrophobic, have been identified for hormones, digestive tract enzymes, and serum proteins. It is postulated that these peptides direct the passage of the nascent polypeptide chain through the membrane of the endoplasmic reticulum. Recently a precursor peptide for an immunoglobulin heavy chain and for several light chains have been characterized. From the limited amino acid sequence data available, the light chain precursors appear to be somewhat homologous within and between subgroups, with much less homology between peptides of kappa and lambda light chains.

In collaboration with A. Williamson and H. Singer (University of Glasgow) we are determining the amino acid sequence of the precursor peptides of a gamma H chain and kappa L chain from a myeloma obtained from a C3H mouse. All reported studies have used material from BALB/c mice. Although neither the heavy nor the light chain of the secreted IgG product have free amino termini, the in vitro synthesized products are not blocked and their sequences can be determined. Preliminary results indicate a distinct lack of homology to the three previously reported kappa L chain precursors, and to the other known heavy chain precursor. The extent of variation of precursor sequence is important in evaluating the role of precursors: 1. in gene rearrangement during development of the immune system, 2. in gene activation, and 3. in product secretion.

Thus far all immunoglobulin precursor peptide investigation has been done using the products of plasmacytomas from inbred mice. It is of interest to establish whether such precursors can be detected in other animals. In the absence of available plasmacytomas, the homogeneous antibody response of rabbits to immunization with streptococcal group C vaccine provides a possible source of a relatively homogeneous immunoglobulin messenger RNA. Consequently, mRNA has been prepared from both the spleen and from the peripheral blood lymphocytes of immunized rabbits. This mRNA has been used in a wheat germ cell-free protein synthesizing system with radioactive amino acids. Specific immunoprecipitation of the product has as yet failed to detect rabbit immunoglobulin among the synthetic products.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00171-01 LIG

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Genetic Studies on Rabbit Immunoglobulins and Other Serum Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Dr. Linda J. Smith	Staff Fellow	LIG NIAID
	Mr. Martin Yarmush	Chemist	LIG NIAID

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.8	0.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The serologic markers of rabbit immunoglobulins-allotypes and idiotypes, are under study because they can provide information concerning the number and arrangement of genes encoding the immunoglobulins. A major effort has been made to characterize allotype $b4^V$, a new allele of the group b allotypes of the C_K region. This allotype, first discovered in this laboratory during amino acid sequence studies, has now been shown by genetic analysis to be an allele of the other group b allotypes and has been characterized serologically. The utility of chain specific idiotypes for genetic studies in the rabbit has been confirmed, and the initial study of an L chain specific idiotype has been extended with an H chain-specific idiotype. It was possible to show linkage of this idiotype to V_H and C_H allotypes within a single allogroup, and to demonstrate latent expression of an allogroup. Genetic polymorphisms of high density serum lipoproteins have been examined by serologic techniques. Structural work has begun on these proteins.

PHS-6040
(Rev. 10-76)

Genetic Studies on Rabbit Immunoglobulins and Other Serum Proteins

The various serologic markers of rabbit immunoglobulins have been valuable probes for the study of genetic interactions involved in the humoral immune response. The rabbit has a uniquely diverse repertoire of allotypes (intra-species antigenic variants), encompassing most regions of the antibody molecule. The structural correlates for some allotypic markers are simple, while for others they are either complex or unknown. Studies within this project are designed to expand the allotypic repertoire, to increase its structural definition, and to use these serologic markers to examine questions about the number of genes involved in immunoglobulin synthesis and the mechanisms controlling interactions among these genes. The studies based on allotypes have been complemented and extended by use of idiotypes as markers for the antibody combining site. The obvious complexity of idiotypes as genetic markers has been lessened somewhat by increasing the specificity of idiotypic antisera, either by fractionation based on antigen competition or by limiting idiotypic recognition to determinants on one of the antibody chains.

Considerable effort has been spent in complete genetic and serologic characterization of a new C_k allotype which was discovered in this laboratory. This allotype, called $b4^v$, is an inherited variant of the rabbit kappa chain allotype b4 and has been characterized structurally by the presence of serine in place of alanine at position 121 and leucine in place of glutamine at position 124. The variant was traced through eight generations of a pedigreed rabbit family. Genetic analysis of this trait demonstrated that it is an allele of the other group b allotypes and recent breedings have produced rabbits homozygous for this light chain type. Two findings, other than the amino acid sequence differences, distinguish $b4^v$ from b4 in our colony. First, the level of expression of $b4^v$ in heterozygous rabbits is less than that of b4. For example, in $b4b4^v$ rabbits, approximately 30% of the preimmune IgG carries $b4^v$. In $b4^vb5$ animals, 46% of the IgG carries the allotype b5, although in animals of allotype b4b5, 38% of the IgG is b5. Second, retrospective analysis of some litters revealed an abnormally low frequency of $b4^v$ in male heterozygotes. On the other hand, male $b4^vb4^v$ homozygotes were found at the expected frequency in prospective crosses between $b4^vb5$ rabbits.

Serologic differentiation between b4 and $b4^v$ has been very difficult because of the structural similarity. In initial studies, using a variety of anti-b4 sera, no difference between the alleles could be found. A serologic distinction between the b4 and $b4^v$ allotypes has now been detected by a radioimmune assay using one of six anti-b4 sera tested. The distinction was enhanced by fractionation of this antiserum on an immunoadsorbent containing cottontail rabbit IgG. IgG with L chains of the $b4^v$ type are deficient in their ability to inhibit binding of b4 IgG to the antibody preparation. Light chains isolated from b4 and $b4^v$ IgG samples could not be distinguished by this assay, indicating that interaction with an H chain is required for expression of the L chain determinants that differentiate b4 from $b4^v$.

The group b allotypes of the rabbit have been used to probe the mechanism underlying uneven utilization of allelic gene products. An almost absolute selection in favor of allotype b4 occurs in the antibody response of heterozygous b^4b^9 rabbits immunized with Group C streptococcal vaccine. A possible explanation for this "allelic selection" in favor of b4 is that during the antistreptococcal response the favored allotype (b4) is associated with V region binding sites yielding higher avidity anti-streptococcal antibodies. In order to test this possibility, IgG from heterozygous, hyper-immune rabbits was fractionated by avidity on a Group C immunoadsorbent column and allotypes of the various fractions were quantitatively determined. Contrary to the predicted result, when secondary immunization sera were examined the fractions of highest avidity were enriched in the nonselected allotype. Antibodies from two primary immunization sera lacked the selection for the non-b4 allotype in high avidity fractions observed for the later sera. Thus, the uneven allotype ratios observed in fractions of different avidity from secondary immunization sera can be interpreted as the result of a secondary maturation effect.

The observations of allelic selection in heterozygous rabbits were extended to rabbits with allotypes b^4b^5 and b^4b^6. Although b4 predominated in immunoglobulin from these rabbits, the allotype ratios in antistreptococcal antibody were the same as those in total IgG before immunization.

Idiotypes can be shown to be inherited in outbred species such as the rabbit. Thus, in addition to the interest inherent in idiotypes as possible points of control in the immune response, idiotypes can, in theory, serve to extend the allotypic repertoire by marking genes encoding the antibody binding site. However, because at least two genes (one for H chain and one for L chain) are necessary for idiotype expression, demonstration of inheritance of idiotypes is possible only when rather sensitive assays are used. The utility of idiotypes as V region markers can be improved considerably by separately examining idiotypic determinants on the two antibody chains. Such chain specific idiotypes have been studied and methods for production of chain specific idiotypic antisera have been developed. Examination of an H-chain idiotype provided evidence for idiotype-V_H allotype linkage within a single haplotype, whenever this haplotype was expressed.

An alternative method of limiting the specificity of idiotypic antisera and manipulating idiotypic reactions is by hapten fractionation of antisera. A hapten, 3-O-α-N-acetylgalactosaminosyl-N-acetylgalactosamine, was found to inhibit the binding of eight idiotypic antisera directed against different homogeneous antistreptococcal Group C polysaccharide antibodies. The sensitivity of the different idiotypic reactions to inhibition by a given concentration of hapten was found to be quite variable, ranging from 20 to 80% inhibition. This result suggested that idiotypic antisera contained two populations of antibodies: those which are reactive with determinants in the antigen-binding site and those directed against nonbinding-site idiotypic determinants. This suggestion was verified by fractionation of idiotypic antisera on affinity chromatography columns consisting of homogeneous antibodies attached to Sepharose. The absorbed idiotypic antibodies were

separated into two populations by successive elution with the Group C hapten and ammonium thiocyanate. The antibody fraction obtained by elution with the hapten comprised approximately 33% of the total idiotypic antibody recovered from the column. The hapten-eluted fractions from different idiotypic antisera varied in their sensitivity to hapten inhibition although each could be inhibited to a greater extent than the original antiserum or the ammonium thiocyanate-eluted fraction. One of the hapten anti-idiotype fractions appeared to be homogeneous by isoelectric focusing whereas the other hapten and ammonium thiocyanate-eluted fractions contained multiple bands. Kinetic analysis revealed that idiotypic reactions of antibody fractions obtained by hapten elution were competitively inhibited by the hapten, although the mechanism by which the competition occurs was not completely resolved by this analysis.

Collaborative efforts are underway with Drs. Anne-Lise Børresen and Kare Berg in Oslo on the high-density lipoproteins of the rabbit. In addition to the medical and biochemical significance of genetic studies of HDL and structural characterization of the apoproteins, the lipoprotein allotypes provide markers for two rabbit chromosomes unlinked to immunoglobulin genes. Progress has been made in structural studies of apoproteins implicated in HDL allotypic variation. Purification and characterization of the H1 1 antigen revealed a polypeptide with molecular weight of 20,000 by gel filtration. The peptide contained 20% neutral sugar and 4.5% neuraminic acid. Amino acid analysis as well as the N-terminal sequence for thirteen amino acid residues and three C-terminal amino acids were determined. Difference index analysis gave an indication that the H1 1 polypeptide is unique.

Purification of R 67 antigen did not give a pure polypeptide in sufficient amount to do chemical analysis. A crude fraction of R 67 antigen had three bands on SDS-PAGE, all with R 67 antigenic activity. The crude fraction contained 10% neutral sugar and 3.5% neuraminic acid. No N- or C-terminal amino acids could be determined for the crude fraction of R 67 antigen.

Publications

Sogn, J.A., Hooke, D.P., Freedman, S.M. and Kindt, T.J. Antibody avidity and allelic selection for L chain allotypes in rabbits hyperimmunized with streptococcal vaccine. J. Immunol. 119: 1024-1028, 1977.

Sogn, J.A., Yarmush, M.L. and Kindt, T.J. Genetic Implications of Linkage Studies Using Chain-Specific Idiotypes. In Sercarz, E.E. & Fox, C.F. (Eds.): Regulatory Genetics of the Immune System, ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. VI. Academic Press, Inc., New York, 1977, pp. 91-97.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00172-01 LIG

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Carbohydrate and Glycoprotein Antigens of Microbial Cell Walls

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous compositional and immunological studies have defined the chemical and antigenic structure of the group-specific carbohydrates for Groups A, A variant and C streptococci. These polysaccharides were shown to have a common core structure. Work is in progress to determine the structure of the group specific carbohydrate of the Group B streptococcus in order to chemically define its antigenic determinants and to determine its chemical relationship to the other streptococcal group specific carbohydrates.

Structural determination of an antigenic polysaccharide isolated from eggs of Schistosoma mansonii is under investigation using GCMS techniques.

Lipoteichoic acid from Staphylococcus aureus was isolated by hot phenol extraction. Amino acid analysis and GCMS methods have yielded data concerning the amino acid composition, fatty acid profile, and carbohydrate composition of this preparation.

PHS-6040
(Rev. 10-76)

Carbohydrate and Glycoprotein Antigens of Microbial Cell Walls

The Laboratory has over the past several years worked on determination of polysaccharide structures. The availability of the expertise in this area has led to the initiation of several collaborative projects. These structural studies involve polysaccharides isolated from the following pathogenic organisms: Streptococcus Group B, Staphylococcus aureus and Schistosoma mansonii. It is an objective of the Laboratory to determine complete structures for these polysaccharides and to relate these structures to biological properties of these organisms.

The group-specific polysaccharide of the Group B streptococcus has been isolated from the $NaNO_2$ extract of lyophilized cells. The composition of this group-specific antigen is identical to that purified by more tedious techniques - 50% rhamnose, 10% galactose and 10% N- acetylglucosamine. In addition, when the composition of the carbohydrates was determined by gas chromatographic analysis of their trimethylsilyl derivatives, a previously unidentified carbohydrate was shown to be present in this polysaccharide. Although its gas chromatographic retention time and anomeric peak distribution has been compared to many known carbohydrates, the newly discovered carbohydrate currently remains unidentified. Other lines of experimentation indicate that the unidentified carbohydrate is acidic in nature.

Analyses of the acetylated derivatives of the permethylated monosaccharides of this group-specific carbohydrate have shown the following sugars to be present:

1-linked rhamnose
1, 2-linked rhamnose
1, 3-linked rhamnose
1, 2, 4-linked rhamnose
1, 3-linked galactose
1, 3-linked N-acetylglucosamine

The Group B streptococcus has been shown to be a major cause of neonatal infections. Information on structural components of its cell wall will help in understanding its pathogenic properties and will provide an aid in classification of the organism.

Current studies are being directed toward identifying the unknown sugar in this polysaccharide and toward determination of the complete structure of the Group B polysaccharide.

An antigenic polysaccharide has been isolated from the eggs of Shistosoma mansonii. Preliminary compositional analysis indicates that the polysaccharide may be a substituted dextran. Planned studies, which include periodate oxidation and methylation analyses, will involve characterization and structural determination of this molecule.

Lipoteichoic acids are typically membrane associated molecules consisting of a glycolipid covalently linked to a long polyglycerol-phosphate backbone. The backbone sometimes displays carbohydrate and D-alanine substituents. Lipoteichoic acid from Staphylococcus aureus was isolated by hot phenol extraction of the dried organisms. Amino acid analysis revealed alanine as the only amino acid in the preparation. GCMS methods have yielded data on the fatty acid profile of the preparation. Future studies will include the determination of the carbohydrate composition and structure and the determination of the chain length of the polyglycerolphosphate backbone. In addition, the nature of glycerol substitution will be determined.

Publications

Coligan, J.E., Fraser, B.A. and Kindt, T.J. Immunochemistry of Streptococcal Group C Polysaccharide and the Nature of its Cross-Reaction with the Forssman Glycolipid. In Marchesi, V.T., Ginsburg, V., Robbins, P.W., Fox, C.F. (Eds.): Progress in Clinical and Biological Research, Vol. 23. ICN-UCLA Symposium on Cell Surface Carbohydrates and Biological Recognition Held at Keystone, Colorado, February, 1977. Alan R. Liss, Inc., New York, 1977, pp. 601-612.

Coligan, J.E. and Slayter, H. Physical, chemical and Immunological characterization of saline-extracted, concanavalin A purified carcinoembryonic antigen. Immunochemistry, in press, 1978.

Coligan, J.E. and Kindt, T.J. Use of Structurally Defined Streptococcal Carbohydrate Antigens in Studies of Rabbit Antibody Idiotypes. In Read, S. and Zabriskie, J. (Eds.): Streptococcal Disease and the Immune Response. Academic Press, New York, 1978, in press.

Coligan, J.E., Kindt, T.J., and Krause, R.M. Structure of the streptococcal groups A, A-variant and C carbohydrates. Immunochemistry, in press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00173-01 LIG

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Nonallelic Expression of Genes Encoding Rabbit Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rabbit immunoglobulin allotypes are antigenic determinants thought to be inherited as autosomal co-dominant alleles. This notion has recently been challenged by observations of low concentrations of allotypes not detected by qualitative tests or predicted by parental genotypes. In the past year, molecules with "latent" allotypes have been isolated from the sera of pedigreed rabbits and shown to be indistinguishable from the normal allotypes. Trivial explanations for these observations, such as cellular chimerism and cross-reactivity of the allotypes, have been virtually eliminated by our recent experimentation. Current research focuses on attempts to describe the role of allotypes in control of immunoglobulin genes and on methods to provoke expression of latent allotypes in vitro and in vivo with various mitogens and other pharmacologic agents.

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In the past year, studies on latent allotypes of rabbit immunoglobulins have included complete serologic characterization of allotypic markers of both the constant and variable regions of the rabbit molecules. It has been shown by precise serologic assays using radiolabeled antigens that the molecules expressing latent allotypes have markers that are indistinguishable from those of the allotypes normally expressed. Future studies in this area will concentrate on determining physiologic conditions necessary to provoke expression of latent allotypes and on control mechanisms that may be involved in the spontaneous expression of the latent allotypes.

Latent Group a allotypes of the V_H region were detected using a radioimmune inhibition assay sera. IgG preparations and antibody fractions containing these allotypes inhibited the binding of insolubilized allotypic antisera to various radiolabeled antigens including IgG pools, homogeneous antibodies, and in the case of a3, a V_H fragment from an a3/b4 IgG. Several different Group a anti-allotype sera were used in the assays and all gave similar results. This indicates that there are no minor serologic determinants which are detected by a certain few of many sera. Comparison of inhibition curves for nominal and latent allotypes indicate that the full spectrum of allotypic specificity may be expressed in latent allotypes.

Hemagglutination and hemagglutination inhibition studies carried out with sera which contained high levels of latent allotypes confirmed the results obtained by the radioimmune assay. It was further indicated that the inhibition values did not, at least in four of the five samples studied in detail, reflect the presence of anti-allotype antibodies in the samples.

In another study, the latent Group b markers of the constant region of the rabbit antibody light chain were detected in sera, in IgG preparations, and on isolated L chains from rabbits bred for homozygosity at the b locus. Serologic analysis of sera from an extended family of homozygous b4 rabbits revealed the presence of latent b allotypes in 5 of 37 sera tested. Latent b5 and b9 markers were identified. None of the sera tested contained latent b6. In two instances, the level of latent b9 allotype was sufficiently high to permit isolation and detailed serologic characterization of the immunoglobulin population bearing this latent allotype. The fact that the latent allotypes were detected in pedigreed, homozygous rabbits minimizes the possibility that lymphoid cell chimerism is involved in latent allotype expression. Furthermore, characterization of the b9 IgG population indicates that the latent allotype determinants do not reside on a subset of molecules with dual allotypic reactivity.

Current studies on the latent allotype concern stimulation of peripheral blood lymphocytes with various B-cell mitogens and analysis of the tissue culture fluid for latent and nominal allotypes. In addition, we are studying the rates of clearance of IgG with different allotypes in rabbits in order to determine whether there is an active clearing mechanism for immunoglobulin molecules expressing allotypes not normally expressed by the rabbit in question.

Publications

Yarmush, M.L. and Kindt, T.J. Isolation and characterization of $b^4 b^4$ IgG molecules expressing latent group b allotypes from pedigreed $b^4 b^4$ rabbits. J. Exp. Med. 148: in press, 1978.

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RESEARCH HIGHLIGHTS

The following sections deal with the more interesting and significant highlights of our research program during the past year. Some of these findings and others were reported in 44 publications. An additional 39 have been accepted by scientific journals.

RICKETTSIOSES

Through collaborative investigations with health departments of many states, we have gained valuable information about the ecology of spotted fever group (SFG) rickettsiae and the disease they cause in man. These studies were continued in New York, Ohio, Texas, Arkansas, and only two new projects, in Oklahoma and Tennessee, were started the past year. In these studies, our primary aim is to secure strains of rickettsiae from ticks and human patients to determine the distribution of serotypes and their relationship to human disease. Ten SFG serotypes have now been found to be indigenous to the United States, but so far, only one has been incriminated as the cause of human disease. (Burgdorfer, Philip)

The efficiency with which Dermacentor andersoni transmits disease agents has long been a subject of interest. During 3 years of study, 7.3%, 1.5%, and 6.2% of 723 ticks attacking man were found to contain Colorado tick fever (CTF) virus, SFG rickettsiae, and SFG-nonstaining bacillary forms, respectively. Through epidemiological surveillance, we learned that less than 50% of persons exposed to CTF-infected ticks acquired the disease and only 2 persons of 11 exposed to SFG rickettsiae contracted spotted fever. None exposed to SFG rickettsiae, other than Rickettsia rickettsii, developed antibodies to these organisms. Although information is fragmentary, it appears that the rickettsia-like organisms prevalent in ticks in this area are not a cause of human disease. (Philip)

The cottontail rabbit has long been incriminated as an effective host reservoir of SFG rickettsiae. However, rabbits inoculated with the virulent "R" strain or Haemaphysalis leporispalustris strain developed only brief rickettsiae of low levels. Tick-rabbit-tick transmission studies indicated poor vector efficiency. Hence, by comparison with other small mammals, such as chipmunks, side-stripes and ground squirrels, the cottontail is a poor host. (Burgdorfer)

Rickettsioses (cont'd)

Continued efforts have been made to serotype rickettsial strains by microimmunofluorescence (micro-IF) tests. So far, 300 strains have been serotyped and one additional serotype, D. variabilis, 369-C-like, has been identified since last year. Three typhus group and 13 SFG rickettsiae have been recognized; of 13 SFG rickettsiae, 10 are indigenous in this country. Of the 10 SFG serotypes, 4 (R. rickettsii, R. montana, R. rhipicephali, and D. variabilis, 369-C agent) are widely distributed in many species of ticks and would be sources of confusion in attempts to identify R. rickettsii by direct examination of hemocytes of ticks taken from patients or in tick surveys. Forty-eight strains of R. rickettsii were similar, regardless of whether they originated from patients or ticks. All of 74 strains of R. montana originated from D. variabilis, D. andersoni, or small mammals from the eastern seaboard states to the Rocky Mountains. R. rhipicephali, originally isolated from Rhipicephalus sanguineus ticks, was common also in D. variabilis, D. andersoni, and D. occidentalis; these were collected in 5 southeastern and western states. Sixty-six strains of the new serotype, D. variabilis, 369-C agent, were recovered from D. variabilis and D. andersoni. In a small ecologic study in the east- and west-side canyons of the Bitterroot Valley, 3 of these serotypes were found to occupy the same ecologic niche in an area of one-half square mile. It now becomes important to clarify the ecologic interactions among these serotypes and the impact of avirulent serotypes on the prevalence of the pathogenic R-type. (Philip, Casper, Burgdorfer)

Two strains each of 4 serotypes were selected for more thorough study of their chemical and antigenic composition. These strains were solubilized and compared by electrophoresis in SDS-polyacrylamide gels. Of 27 protein components found, 21 of lowest molecular weight were common to all 8. However, differences in the components of higher molecular weights served to divide these strains into several groups. Conceivably, these heavier components may be surface antigens that are responsible for their serologic differences. (Anacker, McCaul)

LEGIONNAIRES' DISEASE

Because a practicing physician in Pennsylvania thought psittacosis virus was the cause of Legionnaires' disease, he submitted sera from some of his patients for tests for antibodies against this virus. IgG antibody rises to both the Legionnaires' disease bacterium (LDB) and 6BC psittacosis virus occurred commonly, but IgM antibody rises were restricted to the LDB. These 2 organisms share some antigens, and the serologic diagnosis of these diseases would be confused if specific antibodies were not classified according to immunoglobulin class. The 4 strains of LDB studied varied sufficiently in antigenic composition to constitute 3 serologic types. Their infectivity for guinea pigs varied from 2.1×10^3 to 6.1×10^5 organisms/LD_{50}. In comparisons of the antibiotic sensitivity of LDB and psittacosis virus, we found that rifampin was most effective against both, that erythromycin was moderately effective, and that tetracycline was more effective against psittacosis virus. (Ormsbee)

SYSTEMATICS OF TICKS

Clarification of the taxonomy of groups of medically important ticks remains a major objective of this project. A study of the taxonomic and evolutionary significance of Haller's organ has been made possible by the development of a new cleaning technique that now allows us to obtain unparalleled views with the scanning electron microscope. Haller's organ is used chiefly for chemo- and hygio-receptor activities, and its morphology may be correlated with host-specificity and other behavior characteristics of argasid ticks. This organ was used as one of the means of clarifying the classification of Ornithodoros capensis complex. We have established O. maritimus as a distinct species from O. coniceps, both of which parasitize seabirds. Clarification of this complex is extremely important to understand the distribution of a great variety of viruses they transmit. Similar studies have been made of O. moubata complex whose members are vectors of human relapsing fever in Africa. (Clifford)

Last year, we reported evidence that babesiosis on Nantucket Island was transmitted by a new species of tick, Ixodes dammini, and not by Ixodes scapularis. Besides morphologic differences between the two species, behavioral characteristics, such as the feeding of I. dammini on man, help to separate the two species. I. dammini is now thought to be involved in Old Lyne arthritis, a rather cryptic disease of undefined etiology. In collaboration with the Harvard School of Public Health, we are establishing the distribution of the new species. (Clifford)

ARTHROPOD-BORNE MICROORGANISMS

Projects in this research program involve surveys of field-collected materials for arthropod-borne microorganisms and laboratory studies on the biology of these agents. In past years, we have had difficulty isolating tick-borne viruses in infant mice of the RML strain. This year, we compared 4 strains of mice for the ability to detect virus in 24 frozen tick triturates and in serial dilutions of 9 tick virus stock pools. No significant differences were seen among strains, and all were inferior in sensitivity to Vero cell cultures. We recently completed an evaluation of a newly established cell line from the South African clawed frog for its ability to form plaques with 105 arboviruses and 15 strains of rickettsiae. The Sakhalin group, Uukuniemi and Congo-Crimean hemorrhagic fever group viruses, spotted fever rickettsiae, and certain Wolbachia species formed well-defined plaques in this cell line. Field isolations were not particularly noteworthy this year. A Kemerovo virus and a new member of the Uukuniemi group were isolated from I. uriae ticks taken in Alaska and strains of Sixgun City and Sapphire II viruses were recovered from Argas cooleyi collected in South Dakota. (Yunker, Clifford, Thomas)

Babesia microti, causal agent of human piroplasmosis in Massachusetts, was separated from host-cell components in highly refined form by means of the Ribi-Sorvall cell fractionator. Using this material as an antigen in the micro-IF test, we found it devoid of cross-reactions

with other piroplasms (6 species of Babesia, Anaplasma marginale, and Plasmodium berghi). The carbohydrate component of the organisms appeared to be responsible for species specificity. (Yunker, Kurz, Ribi)

HOST-ECTOPARASITE RELATIONSHIPS

Last year, we defined some of the immune parameters of acquired resistance of guinea pigs to tick infestation. We have now shown that the alternate pathway, not the classical pathway of complement activation, is important in the expression of resistance. Histamine was found not to be involved in resistance, but this substance may be responsible for the itch-scratch response at the site of tick bite, because the level of histamine at sites of tick bites on resistant animals was 5 times that of normal animals. Langerhans cells in the epidermis of resistant animals were found to trap tick salivary antigens. Rabbits developed cross-resistance to other species in a genus, but not to ticks of other genera. Since this resistance response may affect the vector capabilities of ticks, we tested this hypothesis by feeding Francisella tularensis-infected ticks on tick-resistant rabbits, and we found that transmission was blocked. This finding is important in understanding the epidemiology of this disease. (Wikel, Bell)

The resistance of mice to louse infestation was studied by dissecting various aspects of factors involved. Immunosuppressants with known modes of action were shown to delay or reduce resistance and the sequential appearance of cells at immune sites was altered. Skin grafts taken from susceptible and resistance sites of mice, when established on athymic nude mice, developed the same louse burden as they did before transfer. This suggests that a T-cell response is not involved in this resistance. We have shown that this resistance has a genetic basis and breeding experiments are currently underway to establish the nature of this inheritance. (Wikel)

TULAREMIA

Studies on the dynamics of infection of voles exposed by the enteral route have revealed some remarkable findings. Their susceptibility varies as much as 100-fold and these differences are stable. Voles were exposed to large doses of virulent F. tularensis in their drinking water, and at various intervals postinoculation, tissues were taken at necropsy of individual voles. At 1 hour after exposure, the organism was readily isolated from spleen and mesenteric lymph nodes, but then could not be recovered until 2 to 5 days after exposure. It appears that the route of parenteral invasions is through the gut wall, and that penetration of the gut does not depend on propagation in the gut lumen, which was free of organisms after 1 hour. (Bell, Wikel)

RABIES

Continuing our studies on the immunology of rabies, we attempted to explain the occurrence of neutralizing antibodies in some human, bird, and animal populations not overtly exposed to the virus. Among many explanations for these antibodies are the ingestion of infected carcasses, aerosol exposure, and self-limiting infections from a natural exposure. Repeated attempts to induce antibodies in chickens fed large doses of infectious mouse brain failed. Rabies was transmitted to mice by inhalation of aerosolized virus, but sera of all survivors remained free of antibodies. Attempts to stimulate antibodies with sublethal doses given subcutaneously are not complete, but it appears that these naturally occurring antibodies are not related to specific exposure to the virus. (Bell)

PERTUSSIS

Among several studies on the activities of pertussigen, the biologic basis for the induction of footpad swelling of mice has received major attention. When injected subcutaenously into the footpads of mice, crude pertussigen produced a marked swelling in 4 to 24 hours, which subsided the 3rd or 4th day, and the swelling returned again on the 5th to 7th day. This swelling was not prevented by antihistamines, antiserotonins, nor adrenal steroid hormones, and was not complement-dependent. To exclude contaminating endotoxin as the cause of the swelling, we tested endotoxins of Escherichia coli and Bordetella pertussis, but these caused swelling only at 24 hours. However, when 0.002 μg of E. coli endotoxin was given intravenously 24 hours before or 2 hours after pertussigen was given, it blocked both the 4- and 24-hour swelling. Further studies will be necessary to explain the basis of pertussigen-induced swelling. (Munoz)

Pertussis vaccine has been known to be a good adjuvant for stimulating the production of antibody, particularly in the IgE immunoglobulin class. Using purified pertussigen, we are now attempting to determine its mode of action by passive transfer of selected cell populations that have been stimulated and then transferred to syngeneic mice. So far, we have shown that irradiated recipient mice that received antigen and spleen cells of stimulated donors produced IgE antibody, whereas normal spleen cells were ineffective. Our future efforts are directed toward defining the specific roles of T and B cells and macrophages in this system. (Sadowski, Munoz)

Last year, we refuted the reported findings that pertussigen blocks β-adrenergic receptors by showing that this blockade in CFW mice was due to contaminating endotoxins in pertussis extracts. Using RML mice and a nonendotoxin-responder strain, C57BL/10, we found that uptake of ^{3}H-thymidine into the DNA of the isoproterenol-stimulated parotid gland of RML mice was inhibited by endotoxin, whereas that in the latter mouse was not. Whereas pertussigen neither enhanced or blocked the uptake of ^{3}H-thymidine in CFW mice, it enhanced uptake in both RML and C57BL/10 mice, when given

Pertussis (cont'd)

40 to 60 hours before isoproterenol stimulation. Our purest preparations may still be contaminated with endotoxin. These preparations and crude pertussigen retained a large degree of endotoxin activity after heating for 30 minutes at 80°C. (Bergman)

IMMUNOLOGY OF VIRAL AND NEOPLASTIC DISEASES

The following are highlights of research findings of interrelated research projects oriented toward elucidating the pathogenesis of viral diseases and the role of various immune mechanisms in recovery from these diseases. Although principal investigators are indicated, staff involved in these projects collaborate extensively with each other, both in planning and completion of projects.

1. Friend virus (FV) leukemia of mice. We have now established that 3 genes are involved in the pathogenesis and recovery of mice from this disease. Rfv-1 and Rfv-2 are located in the H-2 complex; the Rfv-3 gene is not H-2 associated, but it affects the influence of the first 2 genes on recovery from FV leukemia. In addition to the Rfv-3's influence on FV viremia, it is also associated with antibody-induced loss of FV-cell-surface antigens on leukemic spleen cells 30 to 90 days after virus inoculation. This loss of antigen is associated with increasing cytotoxic antibody to FV leukemia cells, and is reversed when low antigen leukemia cells are transferred to nonimmune irradiated recipients. Genetic back-cross experiments indicate that recovery from viremia, loss of FV cell-surface antigens, and cytotoxicity segregate as a single unit. However, both Rfv-$3^{s/s}$ and Rfv-$3^{r/s}$ mice develop FV-neutralizing antibodies, and the former develop circulating virus-antibody complexes. The appearance of FV-neutralizing antibodies in both genotypes suggests that neutralizing antibody may not be important in recovery, that Ig class or specificity of antibody may not be the same in both, or that antigenic modulation may be involved. (Chesebro, Doig)

In further study of the in vivo defense mechanisms against FV leukemia, we found that H-$2^{b/b}$ mice that had recovered from primary FV leukemia rejected challenge with FV-induced erythroleukemia tumor cells, whereas normal H-$2^{b/b}$ mice were killed. Resistance was evident as early as 6 days after primary FV inoculation, when the primary FV leukemia is growing at an exponential rate and no T-lymphocyte cytotoxic effector cells can be detected. We now plan to label challenge tumor cells to follow the kinetics of their elimination in recovered animals. This information should help to explain the effector mechanisms responsible for recovery from leukemia. (Chesebro)

Previously, we thought that Rfv-$3^{r/s}$ mice could preferentially eliminate the spleen-focus-forming virus (SFFV) from the FV complex that also contains the helper virus (LLV) because we could recover the latter virus from homogenates that did not yield SFFV. Repeating these experiments, we found that 50% of FV-recovered Rfv-$3^{r/s}$ mice retained LLV virus

in their spleens. We have now recovered SFFV from these same mice by transferring spleen cells to FV susceptible H-2 compatible recipients, although transferred cells by themselves were not neoplastic. SFFV was also rescued by cultivation of spleen cells or cell-free homogenates on virus-susceptible SC1 cells. These experiments indicate that persistent infection by both viruses is common and that continued host surveillance is required to prevent relapse of FV leukemia. (Bloom, Chesebro)

2. Dynamics of tumorigenesis. Another leukemia tumor, FBL-3, which originally was derived from a C57BL/6 mouse infected with Friend virus, was studied to determine the basis for modulation of pathogenicity. This tumor expresses only helper virus antigens, and it lacks Ia8 and Thy-1.2 antigens and membrane Ig. When this tumor is maintained intraperitoneally in C57BL/10 mice, subpassage cells taken at 7 days and injected subcutaneously are rejected in 20 to 40 days, but those taken at 14 days kill 50 to 70% of recipients, accompanied by lymphogenous and hematogenous metastases. Through experiments designed to explain the basis of this change in growth potential, we showed that it was not affected by non-neoplastic donor cells in ascites population used for transfer, that the expression of membrane antigens ($H\text{-}2K^b$ and $H\text{-}2D^b$) were comparable in 7- and 14-day cells, that the ability to stimulate blastogenesis of primed syngeneic splenic T-lymphocytes by both 7- and 14-day cells was comparable and that, for the first 10 to 14 days, the growth rates of both types were comparable. Another parameter studied was the production of oncornavirus by regressor (7-day) and progressor (14-day) cell lines, but no significant differences were disclosed. However, others have shown that FV complex is immunosuppressive, and currently, we are exploring the possibility that 7-day and 14-day cells may differ qualitatively in this regard. Clones of both types have been made and, so far, most have behaved like 7-day cells, but one progressor cell line has been established. Further inquiry into the host-defense mechanisms are required to explain the control of growth potential of progressor cells. (Portis)

3. Host defense mechanisms in viral diseases. Extending our findings last year that oil-emulsions of killed Mycobacterium tuberculosis stimulated long-lasting resistance to encephalomyocarditis virus, we have now shown that this resistance is not due to any accelerated clearance of virus from the blood stream, and that resistance was expressed after virus had escaped primary surveillance and had replicated. Splenectomy after injection of mycobacteria reduced resistance, but antiviral resistance was still enhanced if splenectomy was done before. Apparently, mycobacteria localized in areas that were sufficient to enhance resistance, and the spleen was not essential. Similarly, the thymus was shown to be non-essential, because nude and thymectomized mice became as resistant as normal mice. Currently, research efforts are being directed toward elucidating the role of macrophages in this phenomenon. (Lodmell)

4. Role of Ig classes in immune phenomena. This year, major efforts were made to characterize the immune response of hamsters to rabies virus and rickettsiae. Of primary interest was the humoral response of the CNS of hamsters, because a monoclonal response is characteristic of some neurologic diseases of man. However, the antibody response to rabies virus in both the CNS and serum was polyclonal. Similar results were obtained in the examination of sequential CNS/serum samples of dogs and cats affected with recrudescent rabies. In contrast to the selective production of 7S IgG_1 in hamsters immunized with nonadjuvantized purified protein, that induced by rabies was 7S IgG_2. Similarly, hamsters infected with R. typhi mainly produced 7S IgG_2. IgM production to rickettsiae was transient, and no IgA was detected. When antibody fractions were examined for cross-reacting antibodies to other rickettsiae, IgG_2 was more specific than IgM and late IgG_2 was less specific than early IgG_2. The humoral response of Peromyscus maniculatus to infection with R. prowazekii was comparable to that seen in hamsters infected with R. typhi. The role of the immune response on the evolution of rickettsiae and their rodent hosts was examined by comparing the cross-reactivity of infected rodent serums with that of bull frog serums, the evolution of which has not been influenced by rickettsioses. However, the cross-reactivity of bullfrog serums between R. rickettsii and R. prowazekii was comparable to that of hamsters and Peromyscus. Apparently, the immune interactions have not been a factor in the evolution of rickettsiae. (Coe)

The role of various immunoglobulins in immune complex diseases was also studied, particularly those involved in Aleutian disease of mink. For these studies, glomeruli free of interstitial plasma cells were isolated by a special technique that isolates at least 50% of the glomeruli in the kidney. Most affected mink had only complexes of IgA, whereas a few had mixtures of IgM, IgG, and IgA. The predilection for IgA deposition raises intriguing questions about the antiviral specificity of immunoglobulins in this disease, and this problem is presently being studied. (Coe, Portis, Bloom)

PATHOGENESIS OF SLOW VIRAL DISEASES

Several observations during the past few years have strongly suggested that mink encephalopathy virus is, in fact, scrapie virus altered through a single passage in a foreign host. New data add support to this thesis. Goats inoculated 16 months ago with 3rd mink-passage scrapie virus became affected with a disease characterized by a pronounced spongiform change and astrocytosis widespread in the cerebral cortex. This distribution of lesions in these goats is typical of mink encephalopathy and not that of natural scrapie nor that induced by mouse-passage virus of ovine origin. Also, the restriction of virus to the central nervous system of these goats is similar to the distribution of this same virus in mink. Goats inoculated with scrapie virus of sheep origin have virus present in non-neural tissues as well. Finally, the disease induced in mice by scrapie

virus of mink origin is characterized by neurohistologic changes in the cerebral cortex unlike the involvement of subcortical gray masses, brain stem, and spinal cord seen in typical murine scrapie. Genetics of the host also appears to be important. Scrapie virus from Suffolk sheep, but not virus from Cheviot or Montadale sheep, caused encephalopathy in mink. In contrast, virus from Rambouillet and Targhee sheep, which are unrelated to the aforementioned breeds, was highly infectious for mink, but only moderately so for mice. These observations not only emphasize the common identity of scrapie and mink encephalopathy virus but also suggest that difficulty may be encountered in establishing the causal role of scrapie in neurologic diseases of man.

The prevalence of neoplasia in aged mink is interesting, in view of their natural susceptibility to slow viral diseases. Lymphocytic lymphoma is the most common and it appears to be transmissible in young mink. Another neoplasm, fibrosarcoma, which has been passaged through several generations, is more pathogenic for the Aleutian genotype than for the pastel type. (Hadlow, Race)

MICROBIAL COMPONENTS IN CANCER IMMUNOTHERAPY

Although much of this project is basic research on immunopotensive microbial compounds, the clinical application of our findings is an important consideration in our planning. Using the line-10 hepatocarcinoma of strain 2 guinea pigs as a model of human cancer, we have given primary attention to products acceptable in human medicine and the mechanisms by which they enhance tumor immunity. This year, we found that the antigenic adjuvant-active portion of cell wall skeleton (CWS) can be replaced by a simple synthetic analogue resembling a fragment of the mucopeptide moiety of CWS. This substance partially consists of subunits containing N-acetyl-glucosamine-N-acetyl muramic acid and a series of peptide-linked amino acids in the sequence of L-alanine, D-glutamic acid, and M-diaminopimelic acid. Using muramyl dipeptide as the smallest active adjuvant, we found that a dipeptide containing serine was more active than one containing alanine. When serine was replaced by glycine, the muramyl dipeptide had no tumor regressive activity. The specificity of the antigenic amino acid-isoglutamine portion may be important because of probable prior exposure of man and animals to bacteria containing these determinants. These bacterial products appear to act specifically through a T-cell response to antigenic determinants of the adjuvant.

To date, phenol-water or chloroform-methanol extracts of Re mutant Salmonella typhimurium and S. minnesota are the most potent adjuvants. We are now fractionating these endotoxins to isolate less toxic portions that still regress tumors. Of a number of fractions isolated, one, designated B4, is highly toxic but completely devoid of antitumor activity. This loss of activity was related to the removal of amino-acid contaminants from the original Re glycolipid. The tumor regressive potency of B4 was restored

when it was combined with nontoxic side fractions. These fractions are now being examined to determine whether they are structurally similar to active components in BCG CWS. We believe that cross-reactive antigens shared by microorganisms and tumor cells represent immune determinants against which P3, synthetic substitutes thereof, or Re glycolipid augment antitumor immunity. Normal cells likely share the same antigens, but they are probably cryptic where they cannot be recognized. The situation is probably comparable to the antigenic relationship between Re glycolipid and wild-type endotoxin, which is not serologically cross-reactive until hydrolyzed with mineral acids.

Clinical trials of CWS and P3 at the University of Minnesota Hospital and M.D. Anderson Hospital, Houston, Texas, have been encouraging, particularly in the treatment of malignant melanoma. Among fully immunocompetent patients, 83% of injected lesions regressed and 57% of patients with visceral metastases showed improvement. Generally, patients who are nonreactive to a battery of skin-test antigens fail to respond to BCG CWS-P3 therapy. Because of the excellent regressive potency of Re glycolipid-P3 combination in the line-10 guinea pig system, the M.D. Anderson group has obtained FDA approval for Phase I clinical trials with this material. The preparation of these materials at RML also has been approved. (Ribi, McLaughlin)

MECHANISMS OF IMMUNOPOTENTIATION BY MICROBIAL COMPONENTS

The primary aim of this project is to define, at the cellular and molecular level, the mechanisms involved in immunopotentiation effected by microbial components. Previous studies have shown that an effective antigen complex and adjuvant are critical to the process. Further inquiries were made to determine the nature of interactions between these components and whether these components acted independently or synergistically. Extensive dose-response studies revealed that killed mycobacterial whole cells, cell walls, deproteinized cell walls or cell wall skeleton acted synergistically with endotoxin extracts. By appropriate quantitative assays, we showed a correlation between antitumor activity and the ability of mycobacterial cell wall preparations to bind to oil droplets in oil-in-water emulsions. Substances, such as trehalose mycolate, that enhanced this binding augmented antitumor activity. Conceivably, this substance acts by noncovalent lipidation of cell wall skeleton, which, by itself, was thought to interact with oil droplets via covalently-linked mycolic acid residues. We believe these complexes of antigen, trehalose dimycolate, and oil droplets are biologically active depots that slowly release antigen to produce a prolonged stimulus both locally and systemically.

Synergistic antitumor activity of cell wall skeleton combined with endotoxin was also found for oil-in-water emulsions wherein endotoxin was entirely solubilized in the aqueous phase, provided the cell wall skeleton adhered to the oil droplets. Since aqueous endotoxin causes an early

hemorrhagic necrosis of treated tumors, we thought synergism was due to early cytotoxicity. However, in contrast to the inhibition and cellular death induced by mitomycin C, endotoxin does not inhibit cell metabolism (protein, DNA and RNA synthesis) in in vitro systems. Presumably, the early effect of endotoxin on cancer cells is immunologically mediated. (McLaughlin, Ribi)

IMMUNOLOGICAL ACTIVITY OF EUKARYOTIC AND PROKARYOTIC CELLULAR COMPONENTS

Elucidation of the immunologic parameters of cancer immunotherapy is the primary aim of this project. Last year, we showed that systemic tumor immunity, involving the T-cell component, could be transferred from recovered animals to normal animals. Since our supply of strain 2 guinea pigs was drastically reduced the past year, candidate endotoxin preparations were studied also in mouse-tumor models. Only endotoxin preparations that regressed line-10 tumors in guinea pigs induced splenomegaly and an increase in mitogenic activity in cells of endotoxin responder mice. C57BL/10 mice, syngeneic with C57BL/6 mice at the major histocompatibility complex, were nonresponsive to endotoxin products. In view of this finding, we compared the activity of Re glycolipids in endotoxin responder and nonresponder mice affected with established EL-4 lymphomas. Tumors in endotoxin responder mice given chemotherapy and endotoxin immunotherapy regressed whereas similarly treated nonendotoxin responder mice did not. These results indicate that antitumor activity of endotoxin is dependent on the host's ability to respond to the immunostimulant and that the response is genetically controlled.

We also investigated the role of specific immunostimulants (soluble tumor-associated antigens) in the regression of EL-4 lymphomas in mice. Tumor bearing mice given chemotherapy and immunotherapy with soluble KCl extracts regressed tumors, if the soluble antigen was emulsified in minute oil droplets, but not when emulsified in saline. Recovered animals developed specific tumor immunity and the cytotoxic activity of their sera was tumor specific. Similarly, we found that butanol-water extracts of line-10 tumors caused regression of tumors in strain 2 guinea pigs, when the antigen in the butanol phase was mixed with P3 in oil-in-water emulsions. These animals also developed specific tumor immunity. These experiments again demonstrate the important prerequisite of antigen complexed with oil droplets for effective cancer immunotherapy. We plan to elucidate the cellular and humoral responses to antigen presented in this fashion.

Nonviable whole cells of Corynebacterium parvum, which regress some other tumors, was shown to regress a carcinogen-induced leukemia (L-1210) in syngeneic mice. Attempting to isolate and characterize the active components, we found that the phenol insoluble residue fraction regressed tumors of mice treated with chemotherapy, it induced splenomegaly, and had mitogenic activity. Preliminary analyses suggest the active component to be carbohydrate, but further tests are necessary. (Cantrell, Ribi)

STRUCTURE AND BIOLOGICAL ACTIVITY OF ENDOTOXINS

In continued research on the relationship between structure and biological activity of endotoxic components, we have attempted to fractionate endotoxin by various chromatographic methods. So far, we have isolated 3 distinct fractions, B_1, B_2, and B_4, of which only the last was highly toxic and pyrogenic. By itself, B_4 weakly regressed tumors, but, combined with an amino-acid containing side fraction, its tumor regressive activity was fully restored. Repeated precipitation of B_4 with calcium ions removed all tumor regressive activity. This fraction, which was soluble in chloroform methanol, was made water soluble again by removing the calcium ions with EDTA and dialysis against water. The calcium-precipitate endotoxin was resolved into at least 15 bands in an ammonia-containing solvent system, whereas the original material could be resolved into only 4 bands, using the same chromatographic system. The starting material, B_1, B_2, and B_4 fractions, were all mitogenic for LPS responder strains of mice, but only the starting material and a nontoxic side fraction were mitogenic for non-LPS responder strains of mice. These findings demonstrate the complexity of the endotoxin molecule and difficulties in obtaining a nontoxic fraction that regresses tumors.

The discovery of a new strain of mice genetically unresponsive to endotoxin prompted further studies to characterize this strain (C57BL/10 ScN [B-10] of the RML colony). Spleen cells of this strain made typical mitogenic responses to other T and B cell mitogens, but not to endotoxin, regardless of dose or logistics of assay. These mice were resistant to endotoxin in vivo and gave aberrant antibody response to wild type LPS. Lymphocytes of B-10 mice and those of responder strains bound comparable amounts of endotoxin, but cells of the former were not activated. Preliminary experiments suggest that the unresponsive state is controlled by an autosomal recessive gene. (Von Eschen, Ribi)

Annual Report
Rocky Mountain Laboratory
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1977 to September 30, 1978

ADMINISTRATIVE REPORT

As explained in last year's report, any redirection of our research program was delayed until the new Scientific Director of NIAID had the opportunity to review programs and formulate plans for the future. As presently planned, staff of the Rocky Mountain Laboratory will be divided into two laboratories - The Laboratory of Microbial Diseases and The Laboratory of Latent and Persistent Viral Diseases. Projects outside these areas of research will be phased out through attrition of staff, temporary contract support, or other appropriate arrangements.

Dr. Jimmy Williams, Biochemist, transferred from the Naval Medical Research Unit, Bethesda, MD, and is assigned to the Rickettsial Diseases Section. Five nontenured staff appointments were made. Dr. James H. Collins, Staff Fellow, and Dr. William Britt, Research Associate, are engaged in training and research on Friend virus leukemia, under the preceptorship of Dr. Bruce Chesebro. Dr. Yoshiaki Sone and Dr. Hideo Arai, both Japanese scientists appointed as Visiting Fellows, will work with Dr. J. Munoz on fractionation and characterizing of components of Bordetella pertussis. Dr. Jin-ichi Sasaki from Japan was appointed as a Visiting Associate to work with Dr. Ribi on some subcellular aspects of cancer immunotherapy.

During the year, Dr. Robert Smith, Dr. Herbert Hasenclever, and 5 support staff retired. Dr. Kazue Fukushi, Visiting Scientist, completed his research and returned to his post at Hirosaki University, Japan. Dr. Johann Kurz, Visiting Fellow, completed his training and returned to Austria. He is considering several employment opportunities in Austria.

Annual Report
Rocky Mountain Laboratory
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1977 to September 30, 1978

HONORS AND AWARDS

The following activities reflect the recognition afforded staff of the laboratory by their peers in the scientific community.

Awards:

Dr. J. F. Bell received the PHS Meritorious Service Medal for outstanding contributions he made on the immunology of rabies.

Editorial Boards of Journals:

Dr. W. Burgdorfer - Acta Tropica
Dr. C. M. Clifford - Journal of Medical Entomology
Dr. R. A. Ormsbee - Journal of Clinical Microbiology
Dr. Edgar Ribi - Infection and Immunity
Dr. H. G. Stoenner - American Journal of Veterinary Research
Dr. C. E. Yunker - Journal of Parasitology

Drs. J. L. Cantrell, B. W. Chesebro, C. M. Clifford, J. E. Coe, D. L. Lodmell, J. Munoz, and E. Ribi reviewed manuscripts submitted to them by Cancer Immunology and Immunotherapy, Journal of Immunology, Journal of the National Cancer Institute, Journal of Laboratory Animal Science, Annals of the Entomological Society of America, and Tissue Culture Association Manual.

Professional Posts:

Dr. J. L. Cantrell - Elected member, American Association of Immunologists

Dr. B. Chesebro - Member, Immunobiology Study Section, Division of Research Grants, NIH

Dr. W. J. Hadlow - President, American College of Veterinary Pathologists

Dr. H. F. Hasenclever (until his retirement) - Chairman, Standards and Examining Committee for Public Health and Medical Laboratory Mycology, and Member, American Board of Medical Microbiology

Dr. J. Munoz - Trustee of Stella Duncan Memorial Fund for Allergy Research, University of Montana, Missoula

Honors and Awards (cont'd)

Dr. R. A. Ormsbee - Chairman, Subcommittee on Taxonomy of the Rickettsiales, International Committee on Systematic Bacteriology, IAMS

Dr. E. Ribi - Appointed Visiting Professor, University of Texas Cancer System, Texas Medical Center, Houston

Dr. H. G. Stoenner - Board of Directors, Montana Division, American Cancer Society

Dr. S. K. Wikel - Consultant, U.S. Dept. of Agriculture

Dr. C. E. Yunker - Consultant, Michigan State University, Latin American Center, East Lansing; External Faculty Member, W. Alton Jones Cell Science Center, Lake Placid, NY; U.S. Member, International Committee on Polar Arboviruses

Invited Lectures and Participation in Meetings and Symposia:

Drs. R. K. Bergman, C. M. Clifford, J. Munoz, R. N. Philip, R. A. Ormsbee, E. Ribi, and C. E. Yunker participated in internationally sponsored meetings and symposia. Drs. C. M. Clifford, J. Munoz, R. A. Ormsbee, S. K. Wikel, and C. E. Yunker served as organizing chairmen of sectional symposia, most of which were international in scope.

As in previous years, many staff were invited to present lectures at universities, most of which were done in connection with travel to annual meetings of national organizations. Eleven staff members presented invited lectures at 27 different colleges and universities.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00061-16 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Natural History of Tick-Borne Rickettsial Agents and Their Public Health Significance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. Burgdorfer	Res. Entomologist (Med.)	RML NIAID
OTHER:	R. N. Philip	Medical Director	RML NIAID
	R. L. Anacker	Res. Microbiologist	RML NIAID
	L. A. Thomas	Res. Microbiologist	RML NIAID
	E. A. Casper	Nurse Director	RML NIAID
	T. F. McCaul	NIH Visiting Fellow	RML NIAID

COOPERATING UNITS (if any) Dr. J. L. Benach, State of New York Dept. of Health; Mr. P. V. Fournier and Dr. C. Shepler, Texas Dept. of Hlth. Resources; Dr. D. J. Sexton, Okla. City Clinic; Mr. P. Pickett, Arkansas Dept. of Health; Dr. J. Anderson and Dr. L. Magnarelli, Conn. Agricultural Res. Exp. Station.

LAB/BRANCH
Rocky Mountain Laboratory, Hamilton, MT 59840

SECTION
Rickettsial Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3 6/12	6/12	3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with studies of Rocky Mountain spotted fever and other tick-borne rickettsial diseases in the United States regarding ecology, identification, and characterization of the rickettsial agent(s) with their associated vectors. With the collaboration of outside agencies (state health departments, public health laboratories, hospitals, physicans, etc.), source material for comparative studies on the various rickettsial types and information about the distribution of vector species associated with spotted fever in the United States is obtained. This project also considers the cellular and subcellular aspects of interaction between tick-borne agents, particularly rickettsiae, and their vectors.

PHS-6040
(Rev. 10-76)

Project Description:

The continued concern of health officials about Rocky Mountain spotted fever (RMSF) as a public health problem has placed ever-increasing demands upon our research efforts to determine the ecologic factors pertinent to a rational approach to control. Requests for guidance and assistance by outside organizations are important to our research effort in determining the epidemiologic, ecologic, and health impact on the public. Evaluation of field material continues to be our chief source of information about rickettsiae of the spotted fever group (SFG).

Collaborative investigations were continued in New York, Massachusetts, Ohio, Texas, Arkansas, and were initiated in Oklahoma and Tennessee.

Studies on Long Island, New York, have now progressed to the point where most of the screening of ticks for rickettsiae and serologic tests can now be handled by Dr. Benach's group in the State Health Department. This year, 18 suspect cases of RMSF have been reported; 9 were confirmed and one fatality was reported. One confirmed case occurred in New York City proper.

Work in Massachusetts was brought to a close by the untimely death of Dr. Ed Murray. Ten rickettsial isolates from Cape Cod submitted by Dr. William Feng in Dr. Murray's laboratory were serotyped by us as Rickettsia rickettsii (7 strains) and R. montana (3 strains). Two other isolates from ticks were untypable.

Work is ongoing with Dr. Louis Magnarelli and Dr. John Anderson in Connecticut as they continue to screen deer, mice, racoons, and other mammalian sera by microagglutination (MA) tests for evidence of rickettsial activity in Connecticut. Results of the 2-year study being compiled for publication show that low levels of activity by SFG rickettsiae exist in Connecticut. Rickettsia-like organisms were detected in 67 (2.9%) of 2,330 Dermacentor variabilis and 2 (0.4%) of 455 Ixodes scapularis (I. dammini). However, fewer than one-half of the hemolymph test-positive ticks stained with SFG conjugate. Attempted isolation of rickettsiae at RML from 13 of these ticks was unsuccessful. MA tests on 1,093 mammalian sera indicated that raccoons, principally, and white-tailed deer, white-footed mice, and gray squirrels had contact with R. rickettsii. Since 1965, there were only 5 presumptive human cases of RMSF originating in Connecticut.

In Ohio, the Vector-Borne Disease Unit in Columbus has confirmed 8 cases of RMSF, with one fatality this year to date. The prevalence of RMSF was compared in two areas that had comparably-sized human populations that differed in distribution. The disease was more prevalent in areas where the population was diffusely distributed than in areas in which the human population was concentrated in one major urban area.

Studies on survival of rickettsiae in dead ticks were continued. Smothering ticks in mineral oil was used in preference to freezing, as it more naturally simulates what a patient or doctor will do with vaseline or alcohol to the tick. By this approach, loss of viability to the rickettsiae was much diminished because mechanical damage via freezing was not induced. Rickettsiae under these conditions were viable in the tick for as many as 14 days and gave a serologic response in microtus. Studies to determine viability of rickettsiae in blood under various environmental conditions revealed that rickettsiae have remained infectious for up to 6 days at 72° F in clotted and defibrinated guinea pig blood.

We have continued to assess the role of the cottontail rabbit as an effective host reservoir for RMSF rickettsiae. To date, our data show that spotted fever can be induced in cottontails inoculated with the "R" strain; however, the rickettsemia was shown to be of a low level. By comparison, using a typical HLP strain, similar in its antigenic relationship to the "R" strain, lower levels of rickettsia of shorter duration were detected. Transmission studies from tick to rabbit to tick using the HLP strain were very poor. We conclude that the cottontail does not constitute a good host by comparison with other small mammals such as chipmunks, side-stripes and ground squirrels.

Studies using electron microscopy have led to publishable data on the distinguishing characteristics of the cell wall and cytoplasmic ultrastructure of R. rhipicephali from those of R. rickettsii. Investigations pertaining to the ultrastructure and morphological characterization of other rickettsiae, related to but distinct from R. rickettsii, that have been isolated from D. andersoni, D. variabilis, A. americanum, A. marginatum, A. inornata, I. scapularis, and R. sanguineus are under way. Hope of finding definable characteristics useful in taxonomic differentiation of the rickettsial serotypes is our goal.

As a result of our cooperative research activities with outside institutions, we have accumulated many rickettsiae-infected ticks and blood samples from suspected RMSF patients from various parts of the U.S. Much of our future research will be devoted to the isolation and characterization of rickettsial isolates to determine their relationship to R. rickettsii.

The infrequency of the R. rickettsii serotype in D. variabilis and/or A. americanum in areas of high spotted fever incidence raises the question whether R. rickettsii is subject to phase variation or antigenic diversion as a result of host association. Perhaps environmental and physiologic stresses could influence the antigenic makeup of rickettsiae. Such phenomena occur with Coxiella burnetii and certain other bacteria and viruses, but are incompletely known for R. rickettsii. The future course of this project, therefore, will be directed towards clarification of influences of host and/or vector association on the physiology and stability of R. rickettsii and related organisms.

The Texas State Health Department, Region 7, showed by the MA test a high (51%) prevalence of R. rickettsii antibody in patient-associated dogs as compared to 23% in impounded dogs. Similar tests against R. rhipicephali, an avirulent strain of rickettsiae, showed antibodies in 80% of patient-associated dogs versus 74% in surveillance dogs, thus indicating similar exposure of the two groups of animals to R. rhipicephalus-infected ticks.

In Arkansas, 98 ticks taken from pet dogs of persons reported to have RMSF were examined at RML for rickettsiae. Species included 14 D. variabilis, 11 Rhipicephalus sanguineus, and 73 Amblyomma americanum. Hemolymph testing showed that 3 D. variabilis and 20 A. americanum were infected with rickettsia-like organisms. Isolation of rickettsiae in microtus and establishing infected tick-lines by feeding on guinea pigs will be attempted in order to determine rickettsial type present, particularly in A. americanum. A. americanum may be important in the exposure of humans to rickettsia-like agents in Arkansas.

Samples of human blood and ticks being sent from Oklahoma present similar data but a shift in prominent vector is noted, with a greater prevalence of D. variabilis and R. sanguineus ticks as the species most often sent by Dr. Sexton to RML from Oklahoma for screening. Of 272 ticks of 3 species, 24 were hemolymph test-positive. All ticks except 3 unattached A. americanum were from dogs. All ticks with rickettsia-like organisms were put on animals or frozen in beef-heart-infusion broth for isolation attempts. Two isolates from blood specimens have been acquired and are undergoing testing.

Isolation of rickettsiae from ticks (field and patient specimens) from a variety of geographic areas of the U.S. continues in an effort to determine the spectrum and distribution of the various SFG serotypes (see Project No. Z01 AI 00065-05). To date, only R. rickettsii has been isolated from human cases of RMSF. The rarity with which this serotype is isolated from ticks taken from patients or collected in animal and field surveys continues to raise the question of "phase variation" or "antigenic shift" among SFG rickettsiae similar to that in other biological systems.

In work conducted in Germany and RML, hemolymph preparation of some nearly 3,000 D. marginatus were examined by Gimenez and fluorescent antibody (FA) methods for spotted fever and Q fever. Of these, 30 were found to be FA positive for SFG antigens. The D. marginatus ticks were collected from 10 localities in south Germany. Ticks from two of the locations were found to be positive for Q fever. The fact that most members of the genus Dermacentor in the U.S. and elsewhere in the world carry rickettsia-like agents calls for special attention to this genus. The specific host conditions required by this genus for sustaining rickettsiae makes an understanding of these conditions an essential part of the research.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00063-08 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Immune Responses to Rickettsial Infections and Legionnaires' Bacterium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. A. Ormsbee Scientist Director RML NIAID
OTHER: None

COOPERATING UNITS (if any) Dr. R. C. Kimbrough, Good Samaritan Hospital, Portland, OR; Dr. G. A. Dasch, Naval Medical Research Institute, Bethesda, MD.

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SECTION
Rickettsial Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	1	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study immune responses in man and experimental animals to natural and experimental rickettsial infections. Currently, we are studying in guinea pigs the long-term prophylactic qualities of epidemic typhus vaccines, both commercial and experimental. Studies of sera from volunteers previously sensitized to Coxiella burnetii and then given 0.2 µg of Q fever vaccine have involved isolation, purification and characterization of the resulting antibodies by a variety of chemical, physical and immunologic techniques. An investigation of Legionnaires' disease was undertaken because of the unexpected discovery of antigenic relationship between the bacterium of Legionnaires' disease and the 6BC strain of Chlamydia psittaci. The bacterium of Legionnaires' disease was characterized with respect to antigenic relationships with other microorganisms, pathogenicity and limits of infectivity in guinea pigs, behavior in tissue culture systems, antibiotic susceptibility and strain variation.

PHS-6040
(Rev. 10-76)

Project Description:

Our studies on long-term prophylactic efficacy of commercially prepared and experimental vaccines have revealed considerable differences between vaccines against spotted fever and those against epidemic typhus fever. When 4 PD_{50} doses of commercial spotted fever vaccines were used to vaccinate guinea pigs, the animals were found to be almost completely protected (in terms of suppression of fever induced by challenge with live rickettsiae) at 3 weeks after vaccination but not at 13 weeks. In contrast, 4 PD_{50} of commerical epidemic typhus fever vaccine protected guinea pigs against fever resulting from challenge for periods up to 6 months following vaccination. All commercially-produced rickettsial vaccines contained both soluble and particulate antigens, as judged by CF test. Experimental rickettsial vaccines against spotted fever were purely particulate in nature whereas experimentally produced typhus vaccines included a purely particulate preparation as well as others which were mixtures of particulate and soluble antigens. Against spotted fever challenge, the most effective vaccine was the purely particulate preparation. In contrast, the most effective experimental vaccines against epidemic typhus challenge were those comprising both soluble and particulate antigens.

We plan to continue our study of spotted fever vaccine to determine the role that soluble antigen may play in producing transient immunity.

Vaccination with Q fever vaccine usually leaves the recipient with delayed hypersensitivity to the vaccine. Serums from 17 male volunteers who had a history of clinical Q fever or immunization with a Phase I Q fever vaccine 3 to 4 years previously and then had been given an intradermal dose of 0.2 µg of vaccine were studied. Fifteen of the 17 volunteers had responded with typical delayed hypersensitivity reactions at the injection sites. Neither of the nonreactors showed any detectable antibody response. None of the reactors showed any Phase I CF response. Only 6 of the 15 reactors showed any Phase II CF antibody response, most of them with low titers. Eleven of the 15 reactors responded with a moderate Phase II agglutinin (MA) response. All 15 reactors showed a strong Phase I agglutinin response. The IgG and IgM antibodies against Phase I rickettsiae were isolated, purified and examined for protective ability in a mouse seroprotection test. Results indicated that Phase I IgM antibodies were highly protective in the mouse protection test and accounted for more than 90% of the protective capability in the serum. These results suggest that there was a definite immunological memory for IgM in this system.

There are no plans to continue any of these investigations.

Isolates of Legionnaires' disease bacterium were secured from the CDC, the Pennsylvania Department of Health, the Washington State

Health Department, and by isolation at RML from a cord clot from a healthy mother at delivery. Paired human sera were secured from the CDC and the Pennsylvania Department of Health. Antigens for indirect microimmunofluorescence (micro-IF) tests and infectious seeds for experimental infections were made from yolk sacs of infected chick embryos. Highly specific anti-IgG and anti-IgM were employed in the micro-IF tests. Antibiotic susceptibility was determined by plaque inhibition tests in monolayer cultures of primary chick embryo cells. LDB produces well-defined plaques in 6 to 8 days.

Antibody titers of acute and convalescent patients with LDB were measured. IgG antibody rises to both LDB and 6BC were found in some patients, but not in all. However, these patients showed IgM antibody rises against only LDB. These findings suggest that Legionnaires' disease and psittacosis could be confused with one another in the course of routine serologic diagnosis by micro-IF if the specific antibodies are not further characterized by immunoglobulin class.

Differences among the three strains as exemplified by antibody responses of experimentally infected guinea pigs formed the basis of strain characterization. The Pennsylvania (LAP) and CDC (LAC) strains were indistinguishable from each other; strains LAB and LAW were distinguishable from each other and also from the LAP-LAC pair.

Differences in pathogenicity and infectivity are summarized in Table 1. Strain LAW was isolated from a fatal case of Legionnaires' disease and then cultured an unknown number of times before being put back into embryonated eggs in our laboratory. It is clear that it is very much less pathogenic than strain LAC which also was isolated from a fatal case and strain LAB which was isolated from a healthy person. The data in Table 1 are given in terms of number of LDB in the infectious seed as determined by direct count by the method of Silberman and Fiset (J. Bact. 95, 259, 1968).

Table 1. Parameters of infectivity and pathogenicity of three strains of the bacterium of Legionnaires' disease

Test	50% endpoints in numbers of bacteria		
	LAC	LAB	LAW
PFU	7	31	66
$GPID_{50}$	2.1×10^3	$\leq 1.7 \times 10^3$	6.1×10^5
$GPFD_{50}$	4.3×10^3	$\leq 1.7 \times 10^3$	9.4×10^6
$GPLD_{50}$	3.4×10^5	3.5×10^5	$> 7.3 \times 10^8$

Table 2 gives a summary of antibiotic susceptibility tests. Results are in terms of concentrations necessary to inhibit numbers of plaques by 50%. These data make it clear that rifampin is most effective against both LDB and 6BC, that erythromycin is moderately effective against both agents, that tetracycline is much more effective against 6BC than against LDB. The major difference in sensitivity among the strains of LDB is the much greater sensitivity of LAW to rifampin.

Table 2. Antibiotic concentrations which produced 50% plaque inhibition in monolayers of primary chick embryo cells infected with Legionnaires' disease bacterium (LDB) on the 6BC strain of Chlamydia psittaci

	Concentrations in µg/ml of overlay	
	LDB	6BC
Rifampin	3.5×10^{-4} 4.1×10^{-5} (LAW)	2.5×10^{-3}
Erythromycin	3.3×10^{-2}	5.0×10^{-4}
Gentamicin	1.1×10^{-1}	not done
Streptomycin	1.9×10^{-1}	not done
Tetracycline	3.7	2.6×10^{-3}

Future plans include continued investigations of methods of culturing LDB.

Publications:

Ormsbee, R. A.: Qualitative requirements and utilization of nutrients: Rickettsiales. In Rechcigl, M., Jr. (Ed.): CRC Handbook Series in Nutrition and Food. Section D: Nutritional Requirements. Cleveland, CRC Press, Inc., 1977, vol. 1, pp. 27-29.

Ormsbee, R., Peacock, M., Gerloff, R., Tallent, G., and Wike, D.: Limits of rickettsial infectivity. Infect. Immun. 19: 239-245, 1978.

Ormsbee, R. A. and Peacock, M. G.: Antigenic relationships of rickettsiae of the typhus and spotted fever groups. In Kazár, J., Ormsbee, R. A., and Tarasevich, I. N. (Eds.): Proceedings of the 2nd International Symposium on Rickettsiae and Rickettsial Diseases, Smolenice, June 21-25, 1976. Bratislava, VEDA, 1978, pp. 207-219.

Publications: (cont'd)

Peacock, M. G., Fiset, P., Ormsbee, R. A., and Wisseman, C. L., Jr.: Antibody response in man following a small intradermal inoculum of Coxiella burnetii phase I vaccine. In Kazár, J., Ormsbee, R. A., and Tarasevich, I. N. (Eds.): Proceedings of the 2nd International Symposium on Rickettsiae and Rickettsial Diseases, Smolenice, June 21-25, 1976. Bratislava, VEDA, 1978, pp. 593-601.

In press:

Ormsbee, R., Peacock, M., Philip, R., Casper, E., Plorde, J., Gabre-Kidan, T., and Wright, L.: Antigenic relationships between the typhus and spotted fever groups of rickettsiae. Am. J. Epidemiol.

Ormsbee, R. A., Peacock, M. G., Lattimer, G. L., Page, L. A., and Fiset, P.: Legionnaires' disease: antigenic peculiarities, strain differences and antibiotic sensitivities. J. Infect. Dis.

Waldhalm, D. G., Stoenner, H. G., Simmons, R. E., and Thomas, L. A.: Abortion in dairy goats caused by infection with Coxiella burnetii. J. Am. Vet. Med. Assoc.

Stoenner, H. G.: Q fever. In Steele, J. H. (Ed.): CRC Handbook of Zoonoses

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00064-18 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Mechanisms of Phase Conversion in Coxiella burnetii, Causative Agent of Q Fever

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. A. Ormsbee
OTHER: None

COOPERATING UNITS (if any) None

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00065-05 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Antigens and Classification of the Rickettsiae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Anacker	Res. Microbiologist	RML NIAID
	R. N. Philip	Medical Director	RML NIAID
OTHER:	W. Burgdorfer	Res. Entomologist (Med.)	RML NIAID
	E. A. Casper	Nurse Director	RML NIAID
	T. F. McCaul	NIH Visiting Fellow	RML NIAID
	L. A. Thomas	Res. Microbiologist	RML NIAID

COOPERATING UNITS (if any) None

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NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6 6/12	3 6/12	3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-range objectives of this project are to develop practicable procedures for the classification of spotted fever and typhus-group rickettsiae and to determine the nature and biological properties of rickettsial antigens and constituents. Current specific interests are: 1) the serotyping of rickettsiae by microimmunofluorescence, 2) relationship of rickettsiae revealed by toxin neutralization and polyacrylamide gel electrophoresis tests, and 3) nature and properties of rickettsial antigens.

PHS-6040
(Rev. 10-76)

Project Description:

A variety of serological and physicochemical approaches is being utilized to classify rickettsiae within the spotted fever and typhus groups and to isolate and characterize the antigens of these rickettsiae. Included among these procedures are microimmunofluorescence (micro-IF), polyacrylamide gel electrophoresis, and immunoelectrophoresis.

We have continued to serotype rickettsial isolates by micro-IF with particular emphasis on the spotted fever group (SFG). More than 300 strains have now been typed and one additional serotype (Dermacentor variabilis, 369-C-like) has been identified since last year. Currently, 13 SFG and 3 typhus group serotypes are tentatively recognized. Ten SFG serotypes are indigenous to the United States; 6 of these are characteristic of rickettsiae that have been established or proposed as distinct species on the basis of other biologic properties. More information is needed on the significance of the minor differences between closely related serotypes before we attempt to propose a system for classification of SFG rickettsiae based on serologic behavior. At the moment, the serotypes are designated according to species or according to the arthropod host from which the prototype strain was recovered.

At least 4 of the SFG serotypes (Rickettsia rickettsii, R. montana, R. rhipicephali, and D. variabilis, 369-C agent) are widely distributed in the United States and may be sources of confusion in attempts to identify R. rickettsii by direct examination of hemocytes of ticks taken from patients or in tick surveys. All of these serotypes are associated with more than one tick species. To date, we have typed 48 strains of R. rickettsii, R-like, originating in 9 eastern and western states. No differences could be detected among these strains, whether they were isolated from patients or whether they were obtained from 3 tick species (D. andersoni, D. variabilis, or Haemaphysalis leporispalustris). We have identified 74 strains of R. montana from 2 species of ticks (D. andersoni or D. variabilis) and several species of small mammals collected in 13 states from the eastern seaboard to the Rocky Mountains. Thus far, we have detected 83 strains of R. rhipicephali associated with 4 species of ticks (Rhipicephalus sanguineus, D. variabilis, D. andersoni, and D. occidentalis) taken in 5 southeastern or western states. We have also identified 66 strains of the D. variabilis, 369-C serotypes from 2 species of ticks (D. variabilis or D. andersoni) obtained in 4 eastern and western states.

The survey of D. andersoni ticks taken from east- and west-side canyons bordering the Bitterroot Valley initiated last year has been completed. One hundred six isolates of rickettsiae belonging to the SFG were obtained from hemolymph test-positive ticks. The isolates comprise 4 serotypes. Forty-four percent were R. rhipicephali, 39% were the unclassified D. variabilis, 369-C-like serotype, 9% were R. rickettsii,

and 8% were R. montana. There was no particular localization of one serotype to a specific locality. In one instance, 3 of the 4 serotypes were obtained in a collecting site encompassing an area 1/2 mile square. From these findings, it is apparent that rickettsiae comprising these serotypes may share the same ecologic niche.

Prototype strains from the 4 serotypes obtained during the Bitterroot Valley survey are being characterized to determine whether other parameters can be used to differentiate them and to determine the stability of the serologic variations noted by micro-IF. Information obtained to date indicates that the 4 serotypes can be distinguished on basis of pathogenicity in cell culture, embryonated eggs and guinea pigs. Rickettsiae of each serotype give rise to characteristic plaques and CPE in Vero cell monolayers. Prototype isolates of R. rickettsii and R. montana grew well in embryonated eggs and adapted strains were regularly lethal for the embryo on the 4th day. The R. rhipicephali strains could be passed serially in embryonated eggs but grew poorly and even after 8 passages were irregularly lethal for the embryo on the 6th to 9th day. We have not been able to cultivate the 369-C-like strains in embryonated eggs. In guinea pigs the R. rickettsii prototypes gave typical clinical manifestations, occasional deaths and complete immunity against challenge by the virulent Sawtooth ♀2 strain of R. rickettsii. R. montana strains did not give clinical manifestations but did give rise to low levels of micro-IF antibody principally against the homologous strain and partial immunity against R. rickettsii challenge. The same was true of the R. rhipicephali isolates. The 369-C agent appeared not to multiply in guinea pigs and gave no cross-immunity against R. rickettsii. However, primary antibody response against 369-C was reinforced after secondary challenge by R. rickettsii, indicating at least some antigenic relationships between these 2 serotypes.

We have carried prototype strains through 27 to 50 serial passages in Vero cells without change in cultural characteristics. We have not yet rechecked their serological reactivity, but it appears that these serotypes have retained their phenotypic distinctiveness.

In another phase of this project, various properties of 8 strains of spotted fever group rickettsiae isolated from different sources are being studied to determine the variability of these properties and their possible value as aids to identification and classification. Included among these strains are 5 relatively new isolates, 2 well-known strains of Rickettsia rickettsii (the R and Sheila Smith strains), and 1 strain of R. conorii.

In several earlier studies at RML, rickettsial strains were compared according to their ability to induce in guinea pigs antibody which neutralizes the lethal effects of other rickettsial toxins for mice. We simplified and shortened this test so that graded doses of formalinized

and purified rickettsiae are inoculated directly into mice 7 days prior to challenge with the appropriate toxins. For this study, we determined the quantities of rickettsiae of our selected strains required to protect mice against challenge with toxins of R. rickettsii and R. conorii, at present the only toxins available. On the basis of the quantities of rickettsiae required to protect 50% of the mice against challenge with 2 toxins, the 8 strains could be divided into 4 groups. Our results with this limited number of strains indicate that the modified toxin neutralization test can be of value in the characterization of strains of the spotted fever group.

These same 8 strains after solubilization were also compared by electrophoresis in SDS-polyacrylamide gels. Approximately 27 protein components were detected in the different strains by this procedure. All of the strains were greatly similar, if not identical, in their possession of the 21 components of the lowest molecular weights. However, differences in the components of the higher molecular weights (>58,000 daltons) served to divide these strains into several subgroups. Since these strains also exhibit serological differences, it is tempting to speculate that the components of higher molecular weight observed in gels may be the rickettsial surface antigens responsible for the serologic differences.

Evaluation of the hemagglutination (HA) test, employing as antigen alkali-digested rickettsiae purified by density gradient centrifugation, as a diagnostic and research tool was continued. By comparison with results of micro-IF tests of patients' sera performed with labeled anti-IgG and anti-IgM antibody, it was concluded that the HA test detects primarily, if not exclusively, IgM antibody, the globulin which arises early in the course of spotted fever infection. Also, we demonstrated that the HA test, utilizing antigen derived from purified and concentrated rickettsiae, can be used for titrating rabbit and guinea pig antisera. In contrast, other investigators in earlier studies using glutaraldehyde-treated red blood cells and/or a cruder rickettsial antigen were unsuccessful in attempts to measure anti-R. rickettsii antibody in sera from these animals. The HA test, as developed in this laboratory, therefore, may be used clinically for the serological diagnosis of spotted fever in humans (as mentioned in previous reports) and in research for antibody studies involving sera from at least 2 of the most common laboratory animals.

The above studies have demonstrated the considerable variation in properties of the strains of spotted fever-group rickettsiae isolated from nature. Early studies indicated a common characteristic, the ability of each member of the group to fix complement in the presence of antibody to R. rickettsii, but our additional approaches revealed complex interrelationships and differences in reactions with labeled mouse antibody, virulence for laboratory animals, growth in tissue culture and embryonated eggs, cross-immunity tests, toxin neutralization tests, and

polyacrylamide gel electrophoresis. At present, we do not know whether these variations in properties evolved relatively recently from a common ancestor or whether these strains have diverse origins. It is also unknown how many of these strains have the potential for virulence for man. It is hoped that our additional studies will provide a better understanding of these organisms.

In the future, attempts to characterize the antigenic spectrum of selected strains of the SFG by crossed-immunoelectrophoresis will be resumed. Also, efforts will be made to isolate and characterize those antigens which are responsible for protection, group specificity, and species specificity. Certain typhus-group strains will also be included in an attempt to identify the antigen(s) responsible for the sometimes confusing cross-reactions between the typhus- and spotted fever-group agents. If specific antigens can be isolated, an effort will be made to develop serological tests which may be of value for clinical diagnosis of disease or for rapid identification of rickettsial agents.

The presence of at least 3 other SFG serotypes (presumably nonpathogenic for man) in the same tick species in the same localities with R. rickettsii suggest similar ecologic conditions for their maintenance. If the observed variations among serotypes are stable differences (and there is no evidence at present that they are not), then on basis of similar ecologies and immunologic relatedness, nonpathogenic members of the SFG might influence distribution and natural maintenance of R. rickettsii by competition and interference. Experimental studies are initiated to examine this possibility and the findings will be incorporated under Project No. Z01 AI 00061-16.

As opportunity permits, we will continue to selectively serotype by micro-IF isolates from both within and outside the United States with the eventual goal of developing a useful scheme for serologic classification of SFG rickettsiae.

Publications:

Philip, R. N. and Casper, E. A.: Serotyping spotted fever-group rickettsiae with mouse antisera by microimmunofluorescence. A preliminary report. In Kazar, J., Ormsbee, R. A., and Tarasevich, I. N. (Eds.): Proceedings of the 2nd International Symposium on Rickettsiae and Rickettsial Diseases, Smolenice, June 21-25, 1976. Bratislava, VEDA, 1978, pp. 269-279.

Publications: (cont'd)

In press:

Philip, R. N., Casper, E. A., Burgdorfer, W., Gerloff, R. K., Hughes, L. E., and Bell, E. J.: Antigenic typing of rickettsiae of the spotted fever group by microimmunofluorescence. J. Immunol.

Anacker, R. L. and Ormsbee, R. A.: Rickettsiae - general descriptions. In Virology and Rickettsiology, Handbook Series in Clinical Laboratory Science. Cleveland, CRC Press, Inc.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00067-14 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Tularemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Bell	Medical Director	RML NIAID
OTHER:	S. K. Wikel	Staff Fellow	RML NIAID

COOPERATING UNITS (if any) None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4/12	2/12	2/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The dynamics of infection of voles exposed to Francisella tularensis by ingestion (probably the usual route for F. tularensis type B) was investigated. The mesenteric nodes were most frequently infected, implying penetration below the pharynx. Isolations were frequent at one hour, implying that penetration does not depend on proliferation of the organism. In 1945, we reported inhibition of transfer of infection by F. tularensis type A by tick vectors feeding on tick-sensitized hosts. Rabbits become sensitized to ticks that feed on them and subsequent feeding of ticks is ordinarily inhibited. This inhibition, "allergic klendusity", is thereby effective to a measurable degree in preventing transfer of F. tularensis to the host. Effective immunity to infection with fully virulent F. tularensis appears to depend on premunition. The mechanisms involved are complex and have been confused by studies involving bacteria of different degrees of virulence.

PHS-6040
(Rev. 10-76)

Project Description:

There are four objectives in our current studies: 1) to define route of infection and the mechanism of enteral resistance (of voles) to infection with Francisella tularensis, 2) to determine the mechanism of attenuation of virulence of F. tularensis when acquired by the enteral route, 3) to ascertain the effect on transmission of F. tularensis from tick vector to host when the host is sensitized to ticks, and 4) to determine the relationships of specific and nonspecific resistance to infection with F. tularensis bacteria of different virulences.

Enteral route of infection. Infection of voles with F. tularensis by the enteral route and the stable quantum differences in susceptibility to infection by that route have been investigated further. Permeability of the gastro-intestinal tract to F. tularensis differs markedly among members of a population (of voles - Microtus sp.). Susceptibility to enteral route exposure varies by as much as 100-fold among mice. Moreover, that difference is stable. Also, when invasion does occur as a result of enteral exposure, infection is often chronic and nonfatal, whereas parenteral exposure to even one organism causes only acutely fatal infection. We exposed voles by feeding and the site of penetration could, therefore, have been at any point from the oral (even nasal) to the anal mucosa. In recent experiments, voles were exposed to very large doses of virulent F. tularensis by the oral route (drinking); numbers of them were killed at various intervals, necropsied and samples of various tissues were taken for bacterial assay. F. tularensis was isolated commonly from mesenteric nodes and spleen at 1 hour after exposure, but not from cervical, hilar and inguinal nodes, duodenum or colon. Single isolates were obtained from an axilliary node and from pharynx. Subsequently, no cultures were obtained until 2 to 5 days post-exposure when there were increasing recoveries of organisms and generalization of infection. It appears that the route of parenteral invasion is by way of the intestine rather than through the oropharynx and that penetration of the gut wall does not depend on propagation of the bacteria in the gut lumen or in the gut wall structures. Attempts to put this phenomenon on a more easily controllable dose relationship by gavage inoculation did not yield results comparable to the ingestion exposure.

Mechanisms of immunity in tularemia. The mechanisms of immunity to tularemia are poorly understood and are confusing. Much of the confusion results from the fact that various workers have used cultures of F. tularensis of vastly different virulence and what is true of one level of virulence does not hold for another level; e.g., it is quite feasible to vaccinate mice with cell wall vaccine and immunize them against strain #425 (which is virulent for mice, but not for rabbits) but that vaccination is quite ineffective against the fully virulent Schu organism. However, mice vaccinated with cell walls become resistant to Schu if they are first challenged with strain #425. (Premunition, probably.)

Nonspecific stimulation of the RES, effective against another intracellular bacterial pathogen (L. monocytogenes) is not effective against F. tularensis. In vitro studies have demonstrated that humoral factors have no apparent effect on the anti-tularemic activity of the macrophage. However, antibodies may enable a non-specifically activated RES to become effective. That study has been started with F. tularensis strains of defined virulence.

Allergic klendusity (immunity to tularemia as a result of acquired resistance to the tick vector). In the course of experiments on tick transmission of F. tularensis, reported in 1945, we recorded the failure of a large number of infected ticks to transmit infection to a rabbit upon which they fed. Failure was attributed at the time to an allergic reaction of the host, a result of prior exposure to ticks, which inhibited engorgement of ticks in the second exposure. That observation, and the interpretation, have been confirmed in experiments designed to determine efficiency of tick vectors when feeding on naive and on sensitized hosts. Vector efficiency is much greater on the naive hosts. This observation is of great significance to vector-borne disease epidemiology.

Publications:

In press:

Bell, J. F., Wikel, S. K., Hawkins, W. W., and Owen, C. R.: Enigmatic resistance of sheep (Ovis aries) to infection by virulent Francisella tularensis. Can. J. Comp. Med.

Bell, J. F.: Tularemia. In Steele, J. H. (Ed.): CRC Handbook on Zoonoses.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00068-18 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Rabies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Bell	Medical Director	RML NIAID
OTHER:	J. E. Coe	Medical Officer	RML NIAID

COOPERATING UNITS (if any) Dr. D. P. Perl, Dept. of Pathol., Univ. of Vermont, Burlington; Dr. F. A. Murphy, Veterinary School, Fort Collins, CO; Dr. Jared Gardner and Ms. Sally Bauer, Viropathology Section, CDC, Atlanta, GA; Dr. Donald Blenden, Dept. Vet. Microbiol., Univ. of Missouri, Columbia.

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 10/12	10/12	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Currently, there are two major projects on rabies in this section: 1) pathogenesis and immunology of chronic rabies, and 2) evaluation of the significance of rabies virus antibodies in serums of man (unvaccinated) and wild and domestic animals. Our concept of the pathogenesis of chronic rabies and other chronic viral infection of the central nervous system (CNS) envisions a constant monitoring of the CNS by relatively small numbers of lymphocytes, of which only about 1/10 are B cells. A very small fraction of those cells would have receptors for the specific viral antigen, but chance encounter between the appropriate B cell and the infectious focus would lead to proliferation (clone) and antibody formation. T cells (CMI and suppressor cells) are inhibited by virus and are unable to recognize the viral focus because of obscuring antibody. The possible origins of antibodies to rabies virus in unvaccinated people and in wild animals are being investigated.

PHS-6040
(Rev. 10-76)

Project Description:

Major objectives are: 1) to achieve a better understanding of the pathogenesis and immunology of viral infections of the central nervous system (CNS) exemplified by rabies, 2) to resolve some of the paradoxes implicit in much of the literature, viz., the common belief that rabies is an inexorably fatal disease and the contrary belief that frequent occurrence of rabies antibodies in serums of animals and man are the result of sublethal exposures and localized infections to rabies virus.

Because of higher priority assigned to other work we have discontinued studies on the effects of ambient temperature on rabies in mice. The completed work and associated studies on tularemia demonstrated profound effects on infection (10^6 difference in LD_{50} of Francisella tularensis between ambient temperatures of 35° and 15°) in both viral and bacterial infections. These results suggest significant changes in immune responses at different temperatures.

Dr. Donald Blenden now has all of the skin biopsy material requested from us and it is unlikely that further work will be done here. Processing of those tissues is continuing at the University of Missouri.

We are continuing to work with Dr. John Coe on oligoclonal antibodies in chronic CNS rabies infection in several laboratory species and in animals suffering vaccine-induced rabies in veterinary practice. Studies to date, principally in recently recovered hamsters, have not yielded clear evidence of clones; surviving hamsters will be held for a year or two to determine whether sharper separation into clones will develop. Studies on chronically infected cats and long-recovered dogs are not yet at a definitive stage.

Because of approaching mandatory retirement (F. Bell) studies in chronic, recrudescent rabies are being phased out. The results of laboratory studies on two cats with apparent "slow" rabies virus infection in the CNS have been reported at meetings and will be described in detail in a publication.

Rabies and postvaccinal paralysis (PVP) share many signs and symptoms, and in the case of PVP as a result of rabies vaccine, rabies virus antibodies may be found in the serum. Thus, differential diagnosis is sometimes difficult. That difficulty has led to tragic consequences when the PVP syndrome is interpreted as rabies, and vaccination is continued or increased. It may also have led to the common belief that rabies is always fatal because recovery from the syndrome is considered evidence of PVP rather than rabies. We have found the cerebral spinal fluid (CSF) neutralization test helpful in determining whether antibodies are a result of vaccination or of infection. However, it has not been known whether PVP would increase permeability of the blood/brain barrier. In experiments on experimental allergic encephalomyelitis, the experimental equivalent

of PVP, we found that it did not increase permeability of the blood/brain barrier and, therefore, the CSF-neutralization test remains a valid technique for distinguishing between chronic rabies (or recovery from rabies) and PVP.

Significance of serum antibodies in man and animals of unknown exposure history. Serum antibody surveys are routinely used in epidemiology and that precept has been applied to rabies epidemiology, in spite of the fact that rabies is generally considered a fatal disease. In every survey of sera of man and other homiotherms (including birds), some individuals are found to have appreciable titers of rabies virus antibody. Among the many explanations that have been advanced to explain this occurrence are the following: 1) ingestion of infected tissues (especially for birds), 2) aerosol exposure (especially for bats), and 3) subinfectious exposure as by nonlethal bites. In our attempts to resolve the paradox of frequent occurrence of antibodies in serums of unvaccinated animals, we have tested each of the above hypotheses.

Antibodies in bird serums. For this experiment, bantams (Gallus domesticus) were used. They were bled, starved for one day, and then fed a small amount of the usual mash ration mixed with rabies virus-infected (low passage street virus) mouse brain (virus titer [supernatant]: $10^{4 \cdot 2}/0.03$ ml) in various amounts (greatest amount 3 g of brain/hen). The feeding was repeated 3X; blood for serum was taken at 14 days post-feeding on the day of the next successive feeding. Serum neutralization test titers remained nil for all of the 8 test and 2 control hens.

Two weeks after the third feeding all hens were vaccinated by intramuscular injection of 0.5 ml of low-passage infected mouse brain suspension (4 hens) or by intramuscular injection of commercial "Era" vaccine. Two weeks later, all serums gave nil reactions in the neutralization test. Failure of chickens to develop rabies virus antibodies after 3 massive doses of infected brain by the oral route casts doubt upon that source being responsible for antibodies in the sera of wild birds. Failure to respond with antibodies after intramuscular vaccination might have been a result of induced tolerance from feeding (controls fed on uninfected brain were not vaccinated). Otherwise, it is unexplained and will require further test.

Antibodies as a result of aerosol exposure. Rabies as a result of aerosol exposure has been demonstrated repeatedly in the laboratory and in animals exposed in caves. Two human cases have been blamed on cave exposure and several workers have contracted rabies from aerosol exposure in the laboratory. Common occurrence of RV antibodies, especially in bats, is often attributed to nonfatal exposure to the virus in aerosol. As a test of that concept mice were subjected to single and multiple exposures of aerosolized rabies virus suspensions. Mice that died after exposure were tested by the fluorescent antibody technique and were found rabid. Serums

of surviving mice yielded nil reactions in the NT; i.e., antibodies were not elicited by repeated exposures to rabies virus in aerosol suspension.

Repeated sublethal exposure (by bite). Evaluation of this concept could not be done by bite exposures; inoculations were done with needle and syringe. Results of these experiments are not yet available.

Because the principal investigator will retire February 28, 1979, no long-range plans are being made. Primary attention will be given to supplemental experiments needed for completion of research for publication purposes.

Publications:

Bell, J. F., Clark H F., and Moore, G. J.: Differences in efficiency of protective effect caused by high ambient temperature in mice infected with diverse substrains of rabies virus. J. Gen. Virol. 36: 307-315, 1977.

Perl, D. P., Bell, J. F., Moore, G. J., and Stewart, S. J.: Chronic recrudescent rabies in a cat. Proc. Soc. Exp. Biol. Med. 155: 540-548, 1977.

Bell, J. F.: The continuing imperatives of diversity of rabies vaccine. In Hennessen, W. and Regamey, R. H. (Eds.): Proceedings of the Joint WHO/IABS Symposium on the Standardization of Rabies Vaccines for Human Use Produced in Tissue Cultures (Rabies III), Marburg/Lahn 1977. Basel, S. Karger, Develop. biol. standard., vol. 40, 1978, pp. 11-15.

Bell, J. F.: Canine distemper, measles, and SSPE. Am. J. Epidemiol. 107: 354-355, 1978.

Bell, J. F.: Creutzfeldt-Jakob disease and sheep brain (cont.) N. Engl. J. Med. 296: 1415, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00069-47 RML

PERIOD COVERED: October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Systematics and Vector Relationships of Certain Parasitic Arthropods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. M. Clifford	Scientist Director	RML NIAID
OTHER:	J. E. Keirans	Res. Entomologist (Med.)	RML NIAID
	C. E. Yunker	Scientist Director	RML NIAID

COOPERATING UNITS (if any) Dr. H. Hoogstraal, NAMRU-3, Cairo, Egypt; Miss Jane B. Walker, Div. of Vet. Services, Onderstepoort; Drs. D. Sonenshine and Paul Homsher, Old Dominion Univ., Norfolk, VA; Dr. A. J. Spielman, Harvard School of Pub. Hlth., Boston; Dr. D. Stiller, Animal Parasitol Inst., USDA, Beltsville.

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INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4 9/12	1 9/12	3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The activities of this project currently comprise four main functions: 1) Identification of ticks received from various individuals and government agencies throughout the world. Only one other institution in the world is capable of performing this service. 2) Systematic study of certain groups of parasitic arthropods. The foremost tool in systematic studies, the scanning electron microscope, has greatly aided in elucidating taxonomic concepts in acarines actually or potentially involved in transmission of disease agents. 3) Final editing and storage of all RML and NAMRU-3 (Cairo) tick data in the Smithsonian data retrieval system. 4) Colonization of medically important arthropod vectors, which are furnished to outside investigators.

PHS-6040
(Rev. 10-76)

Project Description:

The primary objective of this project is the systematic treatment, including taxonomy and classification, biology, ecology, and colonization, of parasitic arthropods of known or potential medical importance.

Argasid ticks. A major review of ticks of the subgenus Argas of the Neotropical region has been submitted for publication.

A study of the taxonomic and evolutionary significance of the Haller's organ of argasid ticks is underway, in collaboration with Dr. Hoogstraal of NAMRU-3 and Dr. M. Roshdy, an Egyptian investigator. New techniques for cleaning arthropods developed at the RML by Dan Corwin have allowed us to get unparalleled views with our scanning electron microscope (SEM) of this interesting structure. In fact, this cleaning technique will allow us to examine the most minute details on ticks and other arthropods. During the next few years we should be able to revolutionize systematic studies on ticks and solve many of the practical problems that currently confront investigators in this field. Haller's organ is the major one for chemo- and hygio-receptor activities in ticks. We hope to correlate the morphology of this organ with the host-specificity and other behavioral characteristics of the various species of argasid ticks.

A long term study of the Ornithodoros capensis complex of ticks has been underway for the past few years. To date, we have clarified the status of seabird ticks that previously were confused with O. coniceps and resulted in our recognition of O. maritimus as a distinct species. We are redescribing all active stages of O. coniceps and examining several other members of the O. capensis complex as they become available. Studies on and clarification of the status of this group are extremely important to aid us in understanding the distributional significance of the great variety of viruses we have isolated from this tick complex.

During this year, we initiated a study to evaluate the taxonomic status of the O. moubata complex whose members are vectors of human relapsing fever in Africa. In the early 1960's, Dr. Gerald Walton divided O. moubata into several species and subspecies. We are utilizing the SEM to carefully study these various morphological entities proposed by Walton and hopefully produce a practical guide to their recognition.

Ixodid ticks. The review of the adult stages of the genus Ixodes of the Western Hemisphere with over 525 SEM figures has been accepted for publication and we are awaiting reprints. This extremely practical guide to this medically important group of ticks illustrates the possibilities that are available to us with the advent of SEM. It is important to note that, although SEM figures are liberally employed, the

maximum levels of magnifications utilized are well within the range of standard steroscopic microscopes, thereby allowing a wide range of investigators to utilize this guide.

A similar manual to allow for identification of all active stages of the genus Dermacentor is in preparation. This manual should help state health authorities to identify the main vectors of Rocky Mountain spotted fever throughout the United States.

An extensive SEM study of the vector of human babesiosis in New England has resulted in the recognition of a new species of Ixodes. I. dammini ticks recorded as I. scapularis and some nymphal ticks determined as I. muris that were collected north of Maryland have been shown to be this new species. Besides the morphological differences between I. dammini and I. scapularis, behavioral characteristics, such as the fact that the immature stages of I. dammini feed on humans, help to separate these two species. I. dammini assumes even greater importance because it is also presumed to be involved in the rather cryptic disease entity, Old Lyne arthritis. We are continuing studies in conjunction with the Harvard School of Public Health to rear and compare ticks collected at various locations in the Northeast to determine the exact distribution of I. dammini.

Our ongoing investigation of the medically and agriculturally important rhipicephalid species of Africa is progressing as expected. This is a cooperative study with Miss Jane Walker of Onderstepoort. Careful examination with the SEM of all stages of several species reared in the laboratory by Miss Walker has helped to clarify the systematic status of this genus in South Africa. There are definitely more species of Rhipicephalus in South Africa than previous studies had indicated. It is presumed that careful examination of species of this genus in other parts of Africa will provide similar results. These studies are deemed important to allow recognition of the vectors of numerous diseases of cattle in these areas. Currently, ticks and the infectious agents they vector restrict the production of cattle over vast areas of the African continent.

Mite studies. A mange of research laboratory primates is being described in collaboration with workers at Pennsylvania State University. The causal agent is species of mite of the family Psorergatidae and new to science.

RML-NAMRU-3 computer data. The transfer of all the data on the RML and NAMRU-3 tick collections to the Smithsonian computer has been completed. A master list has been prepared and a final edit against the original RML card is nearly completed. Once the editing is completed, the Smithsonian master file will be sorted and structured to allow for more economical day-to-day usage. Then various test runs will be

undertaken and some listings of the ticks in various geographical areas will be prepared. Once it is determined that the data file is functional, access to this resource will be allowed to all interested parties.

Nuttall collection. Dr. James Keirans has spent the last year at the British Museum of Natural History studying the extensive collections of George Nuttall. The Nuttall collection is one of the largest and most interesting collections of ticks in the world. Dr. Keirans has completed his study and we hope to be able to enter these important data in the Smithsonian computer data bank at some future date.

The RML insectary continues to supply tremendous numbers of ticks to investigators both at the RML and at other governmental and private laboratories throughout the world. Last year, over 250,000 live ticks were supplied to these groups. The demand is so great because the RML is the only laboratory in the U.S. that raises enough ticks to be able to supply outside research groups. With the increase in incidence of tick-borne diseases in humans and animals in the U.S., we expect the demand for these services to continue to rise.

The future course of this project will emphasize the use of the SEM to clarify taxonomic problems among various genera of ticks. Special attention will be devoted to the groups of ticks from which we are isolating viruses and other disease agents. We will also stress the work on groups of ticks like the African rhipicephalids that are proven vectors of diseases of humans and other animals. We plan to produce additional practical guides to the identification of ticks that are amply illustrated with SEM photos.

Publications:

Keirans, J. E., Clifford, C. M., and Reddell, J. R.: Description of the immature stages of Nothoaspis reddelli (Ixodoidea: Argasidae) from bat caves in Mexico. Ann. Entomol. Soc. Am. 70: 591-595, 1977.

Clifford, C. M., Walker, J. B., and Keirans, J. E.: Ixodes (Afrixodes) neitzi n.sp. (Acarina: Ixodidae) from the mountain reedbuck in South Africa. Onderstepoort J. Vet. Res. 44: 143-150, 1977.

King, K. A., Keith, J. O., Mitchell, C. A., and Keirans, J. E.: Ticks as a factor in nest desertion of California brown pelicans. Condor 79: 507-509, 1978.

Publications: (cont'd)

In press:

Stark, H. E., Inlao, I., Clifford, C. M., Keirans, J. E., and Lakshana, P.: A preliminary checklist of the ticks of Thailand. Appl. Sci. Res. Corp J. Thailand

Clifford, C. M., Keirans, J. E., Hoogstraal, H., and Morel, P. C.: Observations on the subgenus Argas (Ixodoidea: Argasidae: Argas) identity of Argas (A.) magnus from Peru and Equador. J. Med. Entomol.

Clifford, C. M., Hoogstraal, H., Keirans, J. E., Rice, R.C.A., and Dale, W. E.: Observations on the subgenus Argas (Ixodoidea: Argasidae: Argas). Identity and biological observations of A. cucumerinus from Peruvian seaside cliffs and a summary of the status of the subgenus Argas in the Neotropical faunal region. J. Med. Entomol.

Keirans, J. E., Clifford, C. M., and Hoogstraal, H.: Observations on the subgenus Argas (Ixodoidea: Argasidae: Argas). 16. Argas (A.) moreli, new species, and keys to Neotropical species of the subgenus. J. Med. Entomol.

Keirans, J. E. and Clifford, C. M.: The genus Ixodes in the United States: a scanning electron microscope study and key to the adults. J. Med. Entomol.

Siuda, K., Hoogstraal, H., Clifford, C. M., and Wassef, H. Y.: Observations on the subgenus Argas (Ixodoidea: Argasidae: Argas). 17. Argas (A.) polonicus sp. n. parasitizing domestic pigeons in Krakow, Poland. J. Parasitol.

Spielman, A., Clifford, C. M., Piesman, J., and Corwin, D.: Human babesiosis on Nantucket Island: description of the vector, Ixodes dammini (Acarina: Ixodidae). J. Med. Entomol.

Appendix 1. PL-480 Project 03-063N. Epidemiological and Biological Aspects of Tick-Borne Infections in Egypt and Elsewhere, NAMRU-3, Cairo, Egypt

Annual Funding: $199,679

The new project finally was funded and underway in January of 1978. Under the new agreement, portions of the project will be undertaken at NAMRU-3 and other studies will be performed at Cairo University and the Agouza Arbovirus Unit. The scope of the project has been greatly expanded over that of the previous years.

During the past year, Volume 5, Part 2 of the "Bibliography of Ticks and Tick-Borne Diseases" was completed. This volume contains the published papers in 1974, 1975, and 1976, plus additions and corrections for Volumes 1-5. Work on Volumes 6 and 7, "Bibliography of Tick-Borne Viruses" is progressing nicely and publication is scheduled for 1978 and 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00070-13 RML

PERIOD COVERED: October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biological Activities of Substances in Bordetella pertussis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. K. Bergman	Scientist Director	RML NIAID
OTHER:	J. J. Munoz	Res. Microbiologist	RML NIAID

COOPERATING UNITS (if any) Dr. A. C. Wardlaw, Dept. of Microbiology, Anderson College, University of Glasgow, Scotland.

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study the biological effects of substances from Bordetella pertussis, with special emphasis on those substances that induce physiological and immunological responses in experimental animals. Areas of current investigation are: 1) ability of pertussigen and endotoxin to block β-adrenergic receptors as determined by their effects on uptake of ^{3}H-thymidine in mouse parotid salivary glands, and 2) development of experimental allergic encephalomyelitis in rats after treatment with B. pertussis.

PHS-6040
(Rev. 10-76)

Project Description:

The purpose of this project is to investigate in experimental animals the biological effects of substances from Bordetella pertussis that produce physiological and immunological responses that are related to hypersensitivity and autoimmune phenomena.

We have continued our studies on the ability of pertussigen and endotoxin to affect the uptake of ^{3}H-thymidine into the parotid gland DNA of isoproterenol (IPR)-treated mice. As we reported last year, in CFW mice IPR (a β-adrenergic agonist) induced a striking uptake of ^{3}H-thymidine into the DNA of the parotid salivary gland about 26 hours after IPR was administered. However, if propranolol (a β-adrenergic blocking agent) is given 15 minutes before IPR, the subsequent uptake of ^{3}H-thymidine was blocked. During this past year, we have tested this same system in RML and C57BL/10 mice and found that it worked as well in these two strains as in CFW mice. Similar to results we obtained previously in CFW mice, endotoxin acted in RML mice to block the effects of IPR and prevented the uptake of ^{3}H-thymidine. On the other hand, in C57BL/10 mice, which are highly endotoxin resistant, the administration of endotoxin did not block the IPR induced uptake of ^{3}H-thymidine. Whereas pertussigen neither enhanced or blocked the uptake of ^{3}H-thymidine in CFW mice, it produced a definite enhancement in both RML and C57BL/10 mice when administered 40 to 60 hours before IPR. Thus, these data continue to indicate that pertussigen does not act by blocking β-adrenergic receptors. Even a highly purified preparation of pertussigen administered 24 hours before IPR was ineffective on ^{3}H-thymidine uptake, although it produced histamine sensitivity. Some of our most pure preparations of pertussigen may still have some endotoxin contamination in them. A number of experiments with one preparation showed it to be effective in blocking the uptake of ^{3}H-thymidine in CFW mice when it was administered 90 minutes before IPR. Both crude pertussigen and highly purified pertussigen retained a large degree of this activity even after being heated at 80°C for 30 minutes.

To determine more accurately the efficacy of endotoxins in blocking the uptake of ^{3}H-thymidine, we titrated the effectiveness of three endotoxin preparations. A 16 ng dose of endotoxin from either Escherichia coli or Salmonella enteritidis and a 125 ng dose of endotoxin from B. pertussis were sufficient to induce a significant blockade.

We have attempted during the past year to develop a mouse parotid salivary gland tissue cell culture system in which we could test the effects of pertussigen and endotoxin on the uptake of ^{3}H-thymidine in vitro. To date, we have not been successful in developing such a system. While the fibroblasts have grown very well, we have had very poor success in propagating epithelial cells. We are continuing to work on this problem.

In a continuation of our studies on hyperacute (EAE) in rats, tested the effectiveness of a highly purified pertussigen. The results were interesting because 20 µg of pertussigen administered with 200 mg guinea pig spinal cord to Lewis rats produced a severe encephalomyelitis that killed all rats between 11 to 16 days, although the first symptoms of paralysis did not appear until the 10th day. In contrast, rats that received only 0.8 µg of pertussigen with 200 mg neuroantigen started showing paresis at 6 days. They were all paralyzed and lying on their sides on the 10th day, but they all began to recover and were still alive on the 18th day. Why the smaller dose of pertussigen induced an earlier onset of paralysis than the larger dose is not understood and bears further investigation. We attempted to protect rats from hyperacute EAE by giving them either sera or spleen lymphocytes from syngeneic donor rats that were recovering from EAE. This experiment was unsuccessful. There was no indication that administration of either serum or spleen cells attenuated the development of hyperacute EAE in the recipient rats.

In collaboration with Dr. A. C. Wardlaw of the University of Glasgow, we tested the ability of B. pertussis cells grown in two different media to induce hyperacute EAE. Cells grown in a magnesium enriched medium (C-mode cells) are deficient in many properties, e.g., virulence, immunogenicity, histamine sensitizing factor, mouse protective antigen, etc. as compared to cells grown in a regular medium (X-mode cells). Such was also the case with respect to ability to induce hyperacute EAE. C-mode cells were only 1/10 to 1/100 as potent as X-mode cells.

In future work, we will investigate physiological and immunological similarities or differences which exist between adrenal demedullated mice and pertussigen-treated mice. We are especially interested in comparing blood leukocytes, blood glucose, and blood insulin levels. This may give us a better insight into how the adrenergic nervous system is involved in the mechanism of action of pertussigen. We will pursue our efforts to develop a tissue culture system in which we can better investigate the effects of endotoxin and pertussigen on β-adrenergic receptors. We will also continue to investigate possible mechanisms by which pertussigen enhances the development of EAE in rats.

Publications:

Bergman, R. K., Milner, K. C., and Munoz, J. J.: New test for endotoxin potency based upon histamine sensitization in mice. Infect. Immun. 18: 352-355, 1977.

In press:

Bergman, R. K., Munoz, J. J., and Portis, J. L.: Vascular permeability changes in the central nervous system of rats with hyperacute experimental allergic encephalomyelitis induced with the aid of a substance from Bordetella pertussis. Infect. Immun.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00071-08 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Purification and Activities of Pertussigen - a Substance from Bordetella pertussis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. J. Munoz	Res. Microbiologist	RML NIAID
OTHER:	R. K. Bergman	Scientist Director	RML NIAID
	P. L. Sadowski	Staff Fellow	RML NIAID
	Y. Sone	NIH Visiting Fellow	RML NIAID

COOPERATING UNITS (if any) Dr. W. G. Blackard, Div. of Endocrinology and Metabolism, Medical College of Virginia, Richmond; Dr. A. C. Wardlaw, Dept. of Microbiology, University of Glasgow, Scotland.

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Allergy and Immunology Section

INSTITUTE AND LOCATION

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are to purify, characterize, and study the activities of pertussigen, a substance from Bordetella pertussis. We developed methods to extract and purify pertussigen in sufficient quantities for characterizing the material. By using Triton X-100, BioGel A.5 and ultracentrifugation in glycerol gradients, good yields of highly pure pertussigen were obtained. The mechanism by which pertussigen increases the production of IgE antibodies to hens' egg albumin is under study. The approach employed is to expose mice to pertussigen to see if it has a direct effect on cells involved in the immune response. The response assay involves transfer of lymphoid cells to X-irradiated recipients, which are then primed with antigen. Spleen cells from pertussigen-treated mice produced IgE in recipient mouse primed with an antigen while spleen cells from normal animals did not. The effect of pertussigen on insulin production and the effect of growing B. pertussis cells in media with high Mg on the various biologic activities are also under study.

PHS-6040
(Rev. 10-76)

This year we have concentrated on improving the method of purifying pertussigen by employing a number of significant modifications. As indicated in last year's report, Triton X100 improved the solubility and the recovery of pertussigen during the final step of sucrose density gradient. The improved recovery suggested that Triton X100 might extract pertussigen directly from freshly grown Bordetella pertussis cells, and we found that 0.5% Triton added to 1 M NaCl and 0.05 M sodium pyrophosphate was effective. From 60 to 100% of the activity found in cells has been obtained in a soluble form by this method. From this extract pertussigen was completely precipitated by extensive dialysis in water. This step removed many of the inactive substances that are soluble in water. From the precipitated material pertussigen was extracted by 0.02% Triton in 1 M sodium chloride and 0.05 M sodium pyrophosphate. In this step, nearly 100% of the activity was again recovered. Further purification was obtained by chromatography in a BioGel A.5 column. The active fractions from BioGel A.5 were pooled and concentrated by vacuum dialysis and submitted to ultracentrifugation in a glycerol gradient. The active material was obtained in a fraction with little protein as measured by the phenol reagent. Dr. Yoshiaki Sone is presently trying to characterize the material in this fraction. We have encountered problems in removing Triton X100 from the active fraction and are presently trying to solve this obstacle. Another disturbing finding was that the dialysis tubing releases substances into preparations containing Triton X100 buffer. These two problems have impeded our progress, but we are confident that these minor hurdles will be overcome.

We are presently attempting to produce an antiserum to pertussigen, but so far, as in the past, we have not succeeded in producing an antiserum with strong neutralizing and precipitating activities against pertussigen. These studies are still underway.

In collaboration with Dr. W. B. Blackard from the Medical College of Virginia, we are studying the action of our preparations on the stimulation of insulin production by the B cells of the pancreas. He has shown that the perfused pancreas from pertussigen-treated rats, when stimulated with glucose, produce more insulin than those of untreated control rats. In contrast, the response of isolated pancreatic islets did not seem to be changed.

In collaboration with Dr. A. C. Wardlaw of the University of Glasgow, we have studied the activity of X- and C-mode cells of B. pertussis. C-mode cells are obtained by growing B. pertussis cells in a medium containing high levels of Mg. These cells are inactive in sensitizing mice to histamine, in stimulating IgE production and in increasing susceptibility of rats to experimental allergic encephalomyelitis (EAE). The X-mode cells (Phase I) are fully active in the three parameters just mentioned. The C-mode cells become X-mode cells by simply growing them in medium containing low levels of magnesium.

A considerable amount of work was done on the footpad swelling reaction produced by pertussigen. Although most of the work was done with only partially purified pertussigen, we showed that our purest preparations produced similar response in mice. The following has been observed: 1) A dose of 10 µg of crude pertussigen given subcutaneously into the footpad produces a marked swelling at 4 to 24 hours. The swelling decreases to normal levels by the 3rd or 4th day. On the 5th to 7th days a marked swelling again develops. 2) 20 µg of endotoxin from either B. pertussis or Escherichia coli causes a footpad swelling which is pronounced at 24 hours. This swelling returns to normal by the 3rd to 4th day. No secondary swelling is produced by these endotoxins. 3) 10 µg of our crude pertussigen preparations heated at 80°C for 1/2 hour produced the endotoxin type of swelling but as little as 0.1 µg of a highly purified pertussigen produced the early (4 to 24 hours) as well as the late 5 to 7 days) swelling. 4) An i.v. injection of endotoxin from either E. coli or B. pertussis blocks the early swelling produced by crude pertussigen. As little as 0.002 µg of E. coli endotoxin given i.v. 24 hours before or 2 hours after footpad administration of 10 µg of pertussigen effectively blocked the 4- and 24-hour swelling reaction. Endotoxin was most effective when given at the same time or 1 hour after pertussigen. 5) The swelling produced by crude pertussigen was not prevented by antihistamines, antiserotonins, adrenal steroid hormones or decomplementation of the mice. The reaction was induced in mice genetically deficient in complement. 6) C57BL/10 mice gave only slight 4-hour swelling when endotoxin or crude pertussigen was given by footpad. No reaction at all was observed from 1 to 7 days. With pertussigen, however, these mice responded with a marked swelling at the 6th to 7th day. C57BL/10 mice are known to be unresponsive to endotoxin. Dr. Sadowski has been working on the adjuvant properties of B. pertussis extracts. It is known that pertussis vaccine is a good adjuvant for stimulation of antibody, particularly in the IgE immunoglobulin class, to various protein antigens. The means by which pertussigen stimulates IgE production is being studied by adoptive transfer of spleen cells of inbred C57BL/10Sn mice to X-irradiated recipient mice. Donor mice receive partially purified pertussigen and their spleen cells are transferred to syngeneic mice that have been irradiated to kill all their immunocompetent cells. At the time of transfer, recipient mice are given the antigen. The IgE serum antibody responses of the recipients are then followed by PCA reactions in the skin of normal rats. Dr. Sadowski has shown that 1) Spleen cells from an animal that has received pertussigen produce an IgE response in the irradiated recipient receiving cells and antigen. Cells from spleens of normal animals do not give an IgE response. 2) With semipurified pertussigen, a dose of 1 µg given to the donor mice was most effective while 10 µg or 0.1 µg doses were inferior. The successful transfer of adjuvant activity from donor to recipient was attributable to spleen cells and not to pertussigen adsorbed to red blood cells.

Presently, Dr. Sadowski is trying to find the mechanism by which pertussigen stimulates the donors to produce cells capable of transferring the ability to produce IgE in X-irradiated recipients. The role of B and T cells as well as macrophages is presently under study.

This coming year, Dr. Sone and Dr. Hideo Arai from Japan will be working on this project. Dr. Sone will concentrate in determining the exact chemical nature of pertussigen, and Dr. Arai, who will come in September, will concentrate on determining what activities of pertussis vaccine are due to pertussigen and in finding whether other substances beside pertussigen are involved in protecting mice from intracerebral infection.

We will continue our efforts to produce a good anti-pertussis serum, and a lot of time will have to be spent on producing pure pertussigen for the various studies planned.

We will test our pure material prepared by the latest method for many of the important biological activities, such as IgE stimulation, production of lymphocytosis and leucocytosis, stimulating EAE, mitogenic activity, stimulation of insulin production, toxicity reactions in mice, mouse protective potency, adjuvant action to various antigens, hemagglutinating activity, antigenicity, etc.

Dr. Sadowski will continue his efforts to find the mode of adjuvant action of pertussigen. He will test whether pertussigen has a direct stimulation on lymphocytes involved in IgE production or if its action is on suppressor T cells and macrophages. Suppressor T cells have been postulated by Tada and coworkers to be responsible for controlling IgE production. The mitogenic effects of pertussigen will be studied. Known techniques for separating T and B lymphocytes and macrophages will be used in this work.

Publications:

Munoz, J. J., Bergman, R. K., Cole, R. L., and Ayers, J. C.: Purification and activities of pertussigen, a substance from Bordetella pertussis. In Rosenberg, P. (Ed.): Proceedings of the Fifth International Symposium. Toxins: Animal, Plant and Microbial. Oxford and New York, Pergamon Press, 1978, pp. 1015-1029.

Hasenclever, H. F. and McAtee, F. J.: Antigenic relationships of Candida albicans, Saccharomyces telluris and Saccharomyces cerevisiae. In Lindenmann, J. and Ramseier, H. (Eds.): Contribution to Microbiology and Immunology. Basel, S. Karger, 1977, vol. 3, pp. 126-137

Appendix 1. PL-480 Project 05-042-N. Cytophilic Antibody and Transfer of Skin Reactions, Dr. W. Ptak, Institute of Microbiology, Cracow, Poland.

Annual Funding - $40,126

This project was extended until the end of 1978 without additional funds. The following progress was made since the last report.

Dr. Ptak and Dr. Skowron-Cendrzak have shown that fetal spleen cells completely prevent the development of graft-versus-host syndrome and the transfer of contact sensitivity. Only spleen cells are active. Thymocytes are not active. The suppressor activity of spleen cells disappears early after birth.

Ptak and Rozycka showed that it was possible to induce split tolerance to TNP determinants. TNP-labeled mouse immunoglobulin injected into mice prevented these animals from mounting an anti-TNP antibody response, while the ability to develop contact sensitivity to the same hapten remained unimpaired. The reverse was also true when the mice were given the TNP-labeled autologous cells when only the development of contact sensitivity was decreased. Rozycka and Ptak showed that painting the skin with picryl chloride produces in mice a long lasting suppression of antibody response to the TNP determinant.

Publications:

Ptak, W. and Skowron-Cendrzak, A.: Fetal suppressor cells and their influence on the cell-mediated immune response. Transplantation 24: 45, 1977.

Ptak, W. and Rozycka, D.: Split unresponsiveness to the trinitrophenyl determinant. 1. Maneuvers which suppress either humoral or cell-mediated immune responses. Eur. J. Immunol. 12: 855, 1977.

In press:

Rozycka, D. and Ptak, W.: Split unresponsiveness to the trinitrophenyl determinants. II. Suppression of anti-TNP antibody responses by sensitization with picryl chloride. Immunology

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00072-07 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Host Defense Mechanisms in Viral Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. L. Lodmell Senior Scientist RML NIAID
OTHER: None

COOPERATING UNITS (if any) None

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SECTION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of this project is to delineate the mechanisms of host resistance to viral disease. The principal model for these studies the past 2 years has been an extensive investigation of the humoral and cellular immune functions of mice that exhibit enhanced resistance to a lethal challenge of encephalomyocarditis virus following injection of an oil-emulsion preparation of nonviable Mycobacterium tuberculosis. Additional studies have been initiated to study the pathogenesis and neuroimmunology of rabies virus-infected mice and to ascertain the means by which mice abort central nervous system infections and recover from paralytic disease. In concert with these studies, investigations will be done on the immunological parameters associated with persistent and carrier rabies virus infections.

PHS-6040
(Rev. 10-76)

Project Description:

In general, the major objective of this project is to delineate the mechanisms of host resistance to viral disease. The studies have focused primarily on an in vivo model to investigate the humoral and cellular immune functions of mice that exhibit enhanced resistance to a lethal challenge of encephalomyocarditis virus (EMCV) following injection of an oil-emulsion preparation on nonviable Mycobacterium tuberculosis. Additional studies currently are being initiated to study the pathogenesis and neuroimmunology of rabies-virus-infected mice and to ascertain the means by which these mice abort central nervous system (CNS) infection and recover from paralytic disease.

As a follow-up to our findings of last year that oil-emulsion preparations of nonviable M. tuberculosis stimulate long-lasting resistance to 10 LD_{50} of EMCV, we have now determined that similarly injected mice are resistant to an intravenous or intramuscular challenge with 50,000 LD_{50} of EMCV. The early and rapid clearance of virus from circulation by an activated reticuloendothelial system was considered as a possible explanation for this marked resistance. However, we determined that this is not the case, since virus titers in plasma of normal and mycobacteria-injected mice did not differ at various intervals from 1 to 120 minutes after EMCV injection. In corroboration of these results, it was shown that mycobacteria-injected mice survived EMCV infection, even if the mice became viremic. Thus, means of protection were operative after the virus had escaped primary surveillance mechanism(s) and replicated. Resistance of mycobacteria-injected mice to EMCV also was shown not to be influenced by the mouse major histocompatibility gene complex. In all 8 strains tested, regardless of H-2 genotype, resistance to EMCV was uniform.

Studies designed to determine the mechanism of enhanced resistance by selectively removing or temporarily inactivating various components of the immune system provided several interesting results. It was determined that splenectomy after injection of mycobacteria led to a marked decrease in resistance of mice to EMCV, indicating that resistance was intimately associated with splenic function. If the spleen was removed prior to injection of mycobacteria, however, antiviral resistance was still enhanced. It would appear, therefore, that absence of the spleen led to an alteration in the normal distribution of M. tuberculosis which resulted in localization of mycobacteria in areas that were entirely sufficient to enhance resistance to EMCV. The spleen, therefore, was not the only organ which could harbor effector cells in this system.

The thymus was shown to be nonessential in establishment of enhanced antiviral resistance by nonviable M. tuberculosis in that neonatal mice thymectomized at <48 hours of age, and athymic nude mice became as resistant to EMCV as mice with an intact thymus. Thus, at least one mechanism of resistance to EMCV after injection of mycobacteria appears to involve a

component of the immunologic system other than the T-lymphocyte. At present, we favor the macrophage since studies with a macrophage toxin, silica, indicated that i.p. injected silica completely abrogated resistance of mice to EMCV. These results, in corroboration with preliminary in vitro studies, indicate that there is a strong possibility macrophages may act independently of T-lymphocytes in the enhancement of resistance to EMCV.

The future course of the project associated with induction of enhanced resistance of mice to EMCV by nonviable mycobacteria will focus on a recently developed in vitro assay system. Initial data have indicated that peritoneal exudate cells (PEC) from mycobacteria-injected animals, as compared to PEC from normal animals, can inhibit EMCV replication in mouse embryo cells by >2 $\log_{10}$. Identification of the cell(s) responsible for this inhibition and the mechanism(s) of inhibition will be of major concern. The rabies virus project initially will focus on a comparison of various street virus isolates that proceed to abortive infections in mice and an assessment of the immune response within the cerebral spinal fluid of these animals. The major emphasis of project 00072-07 will gradually shift to a study of the neuroimmunology of CNS viral infections with abortive rabies virus infections of mice as the principal model for investigation.

Publications:

Lodmell, D. L. and Ewalt, L. C.: Enhanced resistance against encephalomyocarditis virus infection in mice, induced by a nonviable Mycobacterium tuberculosis oil-droplet vaccine. Infect. Immun. 19: 225-230, 1978.

In press:

Lodmell, D. L. and Ewalt, L. C.: Induction of enhanced resistance against encephalomyocarditis virus infection of mice by nonviable Mycobacterium tuberculosis: Mechanisms of protection. Infect. Immun.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00073-13 RML

PERIOD COVERED: October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immunity in Mammals and Lower Vertebrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. E. Coe	Medical Officer	RML NIAID
OTHER:	J. F. Bell	Medical Director	RML NIAID
	M. E. Bloom	Medical Officer (Res.)	RML NIAID
	W. J. Hadlow	Res. Veterinarian (Path.)	RML NIAID
	R. A. Ormsbee	Scientist Director	RML NIAID
	J. L. Portis	Medical Officer (SURG)	RML NIAID
	R. E. Race	Vet. Officer	RML NIAID
	L. A. Thomas	Res. Microbiologist	RML NIAID
	S. K. Wikel	Staff Fellow	RML NIAID

COOPERATING UNITS (if any) Dr. Hans Spiegelberg, Scripps Clinic, LaJolla, CA; Dr. Gregory Printz, NIAID, Bethesda, MD; Dr. George Diamandopoulos, Harvard Univ., Boston, MA

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NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 5/12	7/12	1 10/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main objective of this project is to characterize immunological mechanisms in the Syrian hamster and mink, with particular attention to the role played by various Ig classes. Experiments in the hamster utilizing viable organisms (rickettsia, rabies virus) indicate that the antibody response involves a different 7S Ig class than found with nonadjuvantized protein antigens. Lymphomas arising in hamsters, either spontaneously or subsequent to SV-40 inoculation, are being characterized and utilized as sources of monoclonal Ig and cell-class specific antigens. Immunological studies in mink mainly involve elucidating the pathogenesis of Aleutian disease. This immune complex disease in mink appears to primarily involve the deposition of IgA in glomeruli and current ongoing projects are designed to quantify the actual Ig class of anti-Aleutian disease virus and the composition of the circulating complexes.

PHS-6040
(Rev. 10-76)

Project Description:

In collaboration with Dr. J. F. Bell, we have continued our efforts to define the antibody induction mechanisms operative at the central nervous system (CNS) level. Previously, we showed that the response against rabies virus in both the CNS and systemic immune systems was characteristically 7S IgG_2. The oligoclonal and monoclonal CNS antibody responses associated with some human neurologic diseases are of particular interest, not only because of the inductive mechanism responsible for the oligoclonal response, but also because of the potential usefulness of such a technique to develop monoclonal antibodies in experimental animals. The putative CNS antibody of hamsters has been analyzed for electrophoretic heterogeneity by microfractionation on agarose electrophoresis. So far, the anti-rabies CNS humoral response has been polyclonal, with an electrophoretic distribution similar to that found in serum. Similar results have been obtained in examinations of paired CNS/serum samples from dogs and cats with chronic (recrudescent) rabies infections. However, in some cats affected with chronic rabies, the overall electrophoretic mobility of antibody does not reflect the IgG distribution, suggesting that anti-rabies globulin is restricted to a particular Ig class or subclass of Ig. In future studies, we will attempt to explain the observed partially restrictive response in rabies and we will search for oligoclonal responses under theoretical optimized situations, such as minimal or long-term immunization.

Previous studies have shown a predilection for 7S IgG_1 antibody synthesis after immunization of hamsters with nonadjuvantized purified protein antigens. However, 7S IgG_2 antibody was the predominant response to rabies infection. In order to further evaluate the Ig class of antibody made in response to infectious agents, the antibody in hamsters infected with a rickettsia was evaluated for Ig class and specificity. Hamsters were inoculated with Rickettsia typhi and serial samples of serum were examined by indirect fluorescent antibody for antibodies to homologous antigens (R. typhi), and cross-reacting antigens (R. rickettsii, R. prowazekii and R. akari) using specific fluoresceinated antisera specific for hamster IgG_1, IgG_2, IgA and IgM. A transient IgM antibody was detected as early as 5 days after inoculation; Ig antibody was well developed by day 9, and reached maximal titers around day 21. IgA antibody to typhus was not detected and IgG_1 antibody was found in only 1 of 40 sera examined. Comparisons of specificity of IgG_2 and IgM antibody indicated that IgM was less specific than IgG_2 and that late IgG_2 was less specific than early IgG_2. Examination of sera from Peromyscus mouse (P. maniculatus) infected with R. prowazekii showed similar IgG_2 and IgM responses; IgM antibody was detectable 4 days and IgG_2 antibody 6 days after injection. Although endpoint titers greater than 2560 were found in IgM and IgG_2 classes, IgG_1 and IgA antibody were undetectable (<20).

Results to date have indicated that cross reactivity among rickettsial organisms cannot be ascribed to antibody peculiar to a specific Ig class although some quantitative differences in IgM and IgG_2 specificities do exist. The question arose whether antibody specificity for the rickettsiae would be different in a species in which these organisms were noninfective. That is, did the presence of these rickettsiae during the evolution of mammals have a selective influence on mammalian evolution or contrarily, did mammalian immunity have a selective influence on evolvement of serologically defined rickettial groups? Evidence of such an interaction in evolution could be obtained by analysis of cross reactions when antibody was obtained from phylogenetically distant (nonhost) species such as an amphibian. Preliminary cross reactivity measurements of bullfrog antibody to R. prowazekii and R. rickettsii indicate that high titered antisera can be produced in bullfrogs and the specificity appears similar to that found in immune mouse serum. Bullfrogs are also being used as a source of antibody against human and rabbit immunoglobulins in order to detect determinants which are empirically nonantigenic in other mammals.

We have continued our study of the Ig class(es) involved in the immune complex glomerulitis of Aleutian disease and have now surveyed the kidneys of 28 sapphire mink inoculated with Pullman isolate of Aleutian disease virus. Twenty one of these animals showed exclusive deposition of IgA in the capillaries and mesangeal cells of glomeruli. Five mink had glomerular deposits consisting of various combinations of IgG, IgA, IgM, but IgA was invariably the most prominent Ig.

We are currently attempting to elute Ig from the kidneys to confirm the predilection for IgA deposition and to gain information on the antigen specificity of the Ig deposited in the glomerulus. Because of interstitial collections of plasma cells, it has been necessary to examine isolated glomeruli. In collaboration with Drs. Portis and Wikel, a simple rapid method for glomerular purification has been developed, using isopyknic low speed centrifugation. The technique requires no special equipment and yields from 30 to 50% of the total glomeruli in the cortex with >95% purity. Ultrastructural studies reveal no significant disruption of normal structures except for mild to moderate swelling of endothelial cells at the periphery of glomeruli. These isolated glomeruli will be used to quantify the class of Ig found in kidneys of resistant (pastel) and susceptible (sapphire) AD mink using a radioimmunoassay and a hemagglutination inhibition technique.

The predilection for IgA deposition in glomeruli raises intriguing questions about the Ig class actually containing the anti-ADV and the composition of the circulating complexes. Both of these problems are currently being examined - the former by indirect FA with specific fluoresceinated anti-mink IgG, IgA and IgM antisera on ADV infected Crandall tissue culture cells and the latter by assay of the composition of the circulating complexes. In addition, the basic cellular responsiveness of mink will be evaluated during the course of AD.

We have previously demonstrated the synthesis by a SV-40 induced lymphoma of a monoclonal 7S IgG_2 protein that was also present as membrane bound Ig. We have now examined 4 other SV-40 induced lymphomas which we received from Dr. George Diamandopoulos, and other lymphomas that spontaneously appeared in our outbred and inbred hamsters (normal and experimental). Of the 5 SV-40 induced lymphomas, all but one were composed of B cells with IgG_2 in the membrane (IgG_2 B cells). This association between SV-40 oncogenesis and IgG_2 B cells raises the question of an SV-40 receptor present predominantly on IgG B cells or a propensity for these cells to be the end product of SV-40 transformation. A hamster plasmacytoma, described earlier by Fortner, has been found to be secreting an IgA and has membrane bound IgA. In addition, we have isolated an IgA secreting lymphoma, which spontaneously appeared in normal LSH hamsters. These SV-40 and spontaneous derived hamster lymphomas are important as a source of monoclonal Ig and also provide information about hamster B cell physiology. They also serve as a source of B cell (and possibly T cell) antigens for induction or absorption of T and B cell specific antibody.

Future work will expand and develop the various projects presented herein. Particular emphasis will be placed on Aleutian disease of mink. In collaboration with Drs. Bloom, Portis and Race, we will continue studies on the pathogenesis of immune complexes, the Ig class responding to ADV, the cellular responses in mink with and without disease. Many of these studies will utilize the color phase (pastel vs. sapphire) susceptibility to Pullman ADV, a model developed by Drs. Eklund and Hadlow; defining basic differences in immunology and/or physiology of these two types of mink may explain the differences in susceptibility to ADV. Schistosmiosis is known to produce immune complex disease in hamsters, and in collaboration with Dr. Wikel, the Ig class composition of these complexes and their relative pathogenicity in various forms of disease will be evaluated. The CNS response to rabies in hamsters, cats and dogs will be analyzed in collaboration with Dr. J. F. Bell and studies on rickettsial serology in lower vertebrates will continue with Dr. Ormsbee.

Publications:

Coe, J. E. and Race, R. E.: Ontogeny of mink IgG, IgA, and IgM. Proc. Soc. Exp. Biol. Med. 157: 289-292, 1978.

Coe, J. E.: Humoral immunity and serum proteins in the Syrian hamster. Fed. Proc. 37: 2030-2031, 1978.

Portis, J. L. and Coe, J. E.: Functional studies of membrane-bound immunoglobulin on hamster macrophages and mast cells. Fed. Proc. 37: 2034-2036, 1978.

Publications: (cont'd)

Leong, D. and Coe, J. E.: Metabolism of homologous and heterologous serum proteins in garter snakes (Thamnophis ordinoides). Immunology 34: 931-937, 1978.

In press:

Coe, J. E.: Serum proteins: normal values derived from normal strains. FASEB Data Book - Inbred and Genetically Defined Strains of Laboratory Animals

Portis, J. L. and Coe, J. E.: Histamine release from mast cells of the Syrian hamster. Immunology

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00074-06 RML

PERIOD COVERED: October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Host Defense Mechanisms in Chronic Viral and Neoplastic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. W. Chesebro	Medical Officer (SR SURG)	RML NIAID
OTHER:	J. K. Collins	Staff Fellow	RML NIAID
	M. E. Bloom	Medical Officer (Res.)	RML NIAID
	J. L. Portis	Medical Officer (SURG)	RML NIAID

COOPERATING UNITS (if any) Donald Doig, Dept. of Microbiology, Montana State Univ., Bozeman; Dr. Kenneth Watson, Dept. of Chemistry, Univ. of Montana, Missoula.

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main goal of this project is the elucidation of host defense mechanisms involved in recovery from virus-induced leukemia in mice. We have approached this problem by attempting to separate various components of the host response against leukemia by identifying individual genes which influence different aspects of this response. Three new genes (Rfv-1, Rfv-2, Rfv-3), which influence recovery from established Friend virus leukemia in mice, have been identified. We now have information concerning some of the immune mechanisms influenced by these genes and the interactions that occur among these mechanisms.

PHS-6040
(Rev. 10-76)

Project Description:

We have identified 3 new mouse genes (Rfv-1, Rfv-2 and Rfv-3) that are involved in host defenses against Friend virus (FV)-induced erythroleukemia. The Rfv-1 gene is located in H-2D region, and has a major influence on the incidence of recovery from established leukemia. The Rfv-2 gene is also associated with the H-2 complex (K, I or Tla region), and has a weaker effect on recovery from leukemia. The Rfv-3 gene is not associated with H-2; however, it is necessary for H-2-associated (Rfv-1 and Rfv-2) effects on recovery from FV leukemia. In addition, the Rfv-3 gene has a strong influence on the ability of certain mouse strains to recover from FV viremia. This effect is independent of the H-2 genotype and can occur even in the presence of progressive leukemic splenomegaly. Thus, by genetic manipulation, 3 distinct components of the host defenses against FV and FV leukemia have been isolated. Current efforts are aimed at further characterization of the cell types and organs involved in these genetically controlled defense mechanisms.

Rfv-3 gene effects. In addition to its influence of FV viremia, the Rfv-$3^{r/s}$ genotype is associated with the antibody-induced loss of FV-cell-surface antigens on leukemic spleen cells 30 to 90 days after virus inoculation. This antigen loss is specific for FV-induced antigens and amounts to an 80% reduction in FV antigens, as measured by quantitative absorption of cytotoxic antibodies. In kinetic experiments, loss of FV antigen is associated with a rise in titer of cytotoxic antibodies specific for FV leukemia cells, and is reversed when low antigen leukemia cells are transferred to a nonimmune splenic environment. Genetic backcross experiments indicate that recovery from FV viremia, loss of FV cell surface antigens, and presence of cytotoxic antibodies segregate together as a single unit. However, the presence of anti-FV neutralizing antibodies does not follow this pattern, as both Rfv-$3^{s/s}$ (viremic) and Rfv-$3^{r/s}$ (nonviremic) mice have neutralizing antibodies, when a sensitive complement-mediated neutralization test is used. Rfv-$3^{s/s}$ mice appear to have persistent circulating infectious virus-antibody complexes in their plasma. The mechanism of the Rfv-$3^{r/s}$ effect on viremia and FV cell surface antigen loss is still unclear. The presence of anti-FV neutralizing antibody in both Rfv-$3^{s/s}$ and Rfv-$3^{s/r}$ mice suggests either that neutralizing antibody is not important in loss of FV antigens and control of FV viremia or that the Ig class or specificity of the neutralizing antibodies made in different mice could be different, leading to divergent biological results. On the other hand, the strong association between Rfv-$3^{r/s}$ effects on viremia and antigen loss and the presence of cytotoxic antibody to FV leukemia cells suggests that antibody-leukemia cell interactions may be responsible for both FV antigen loss from leukemia cells (antigenic modulation) and also decreased virus production in the spleen, which results in decreased levels of circulating virus. We are attempting to characterize circulating infectious immune complexes from Rfv-$3^{s/s}$ mice, with the particular aim of demonstrating whether these complexes have a

shorter circulating half-life in Rfv-$3^{r/s}$ mice than in Rfv-$3^{s/s}$ mice. Also spleen and bone marrow chimeras between Rfv-$3^{s/s}$ and Rfv-$3^{r/s}$ mice are being made to study cell types and organs involved in the Rfv-3 effect on viremia.

H-2-associated genes (Rfv-1 and Rfv-2). We have now examined the influence of two additional H-2 alleles on recovery from FV leukemia. H-2^{s} is associated with high recovery incidence and H-2^{q} with low recovery incidence. In the case of these alleles as well as for the alleles previously studied (H-2^{d}, H-2^{a}, H-2^{b}) the effects of each allele on FV leukemia are opposite to their effect on Kaplan-RadLV induced leukemia. Since both FV and Kaplan-RadLV are influenced by a gene in the H-2D region, it is unclear if these opposite effects are due to the presence of separate genes within H-2D or whether the gene products of the individual H-2D alleles interact with these two viruses in different ways leading to different effects on recovery from leukemia. We are currently attempting to make a RadLV pseudotype of FV to see whether the pattern of H-2-associated recovery will be that of RadLV or the original FV. This should provide evidence concerning which viral component is involved in the H-2D-associated recovery phenomenon.

Mice that recover from FV leukemia have cytotoxic T-lymphocytes in their spleen and bone marrow that can lyse FV leukemia cells. These T-lymphocytes may be the effector cells responsible for recovery from leukemia. Using four approaches, we are now trying to characterize the viral antigens recognized by these T-lymphocytes: 1) isolation of plasma membranes and molecules from plasma membranes that can inhibit the virus-specific T-cell mediated cytotoxic reaction (CMC), 2) inhibition of CMC by antisera with specificity for various virus-induced cell-surface antigens, 3) in vitro stimulation of CMC effector cells by plasma membranes, and 4) characterization of virus-induced antigens on a group of FV leukemia cell lines and correlation of these antigens with ability of these lines to serve as targets for CMC.

In current experiments we are making irradiation chimeras between H-$2^{b/b}$ (high recovery) and H-$2^{a/b}$ (low recovery) mouse strains to see which organs and cell types are responsible for the H-2-associated recovery effects. Previously we found that lethally irradiated H-$2^{b/b}$ mice that received syngeneic spleen and bone marrow cells had a low incidence of recovery from high FV doses, when compared to unirradiated mice of the same strain. The reasons for these differences are not known, and we are now studying the immune response to nonviral antigens and also following the kinetics and magnitude of virus infection after FV inoculation of these mice. Recent experiments have suggested that these irradiated reconstituted mice can recover from lower FV doses, and if this finding is reproducible, comparison of the transfer of cells of different H-2 genotypes should be feasible.

We have also studied in vivo defense mechanisms against FV leukemia cells by challenge of mice with an FV-induced erythroleukemia tumor cell line (Y101F). H-$2^{b/b}$ mice that have recovered from primary FV leukemia can reject these tumor cells; however, mice that have not been inoculated previously with FV are killed by the tumor. Challenge of FV inoculated mice with Y101F cells indicates that resistance can be detected as early as 6 to 9 days after FV inoculation. At this time, the primary FV-induced leukemia is still growing at an exponential rate and no T-lymphocyte cytotoxic effector cells can be detected. Future experiments will be aimed at labeling of the tumor cells with ^{3}H-thymidine or ^{125}I-IUDR to determine the exact time of elimination of these cells during the course of recovery. This should provide more information on the effector mechanisms responsible for in vivo recovery from leukemia.

Persistent FV infection after recovery from leukemia. Recent experiments suggest that Rfv-$3^{r/s}$ mice can preferentially eliminate the spleen focus-forming virus (SFFV) component of the FV complex, compared to the helper lymphatic leukemia virus (LLV). It is unclear whether this different pattern of elimination of infection is due to antigenic differences between SSFV and LLV or to other biological differences (i.e., different cell types infected, etc.). Previously this difference in clearing of the SFFV and LLV was seen in spleens of leukemic Rfv-$3^{r/s}$ mice 30 to 90 days after FV inoculation. We have now tested the spleen homogenates from a group of mice that had recovered from FV leukemia, and we observed persistent LLV infection in 50% of the mice. Transfer of the spleen cells of these mice to FV susceptible H-2 compatible recipients indicated that SFFV could be "rescued" from these cells in a nonimmune environment, although the transferred spleen cells themselves did not have neoplastic properties. SFFV was also "rescued" by cultivation of the spleen cells from recovered mice in vitro with virus-susceptible SC1 cells. In some cases SFFV was detected by infection of the SC1 cells with the cell-free spleen homogenate. Because of these latter results the "rescue" of SFFV from recovered spleen cells may really be the amplification of a low grade persistent SFFV infection in spleen cells after recovery from leukemia. These experiments appear to indicate that persistent infection by both LLV and SFFV is not uncommon and continued host immune surveillance may be required to prevent relapse of FV leukemia.

Publications:

Chesebro, B., Wehrly, K., Watson, K., and Chesebro, K.: Murine leukemia virus infectious centers are dependent on the rate of virus production by infected cells. Virology 84: 222-226, 1978

Chesebro, B. and Wehrly, K.: RFV-1 and Rfv-2, two H-2-associated genes that influence recovery from Friend leukemia virus-induced splenomegaly. J. Immunol. 120: 1081-1085, 1978.

Publications: (cont'd)

Chesebro, B., Wehrly, K., and Housman, D.: Lack of erythroid characteristics in Ia-positive leukemia cell lines induced by Friend murine leukemia virus: brief communication. J. Natl. Cancer Inst. 69: 239-242, 1978.

Chesebro, B. and Wehrly, K.: A single non-H-2 gene is required for H-$2^{b/b}$-associated recovery from Friend virus leukemia in mice. In Bentvelzen, P., Hilgers, J., and Yohn, D. S. (Eds.): Advances in Comparative Leukemia Research 1977. Amsterdam-New York, Elsevier/ North-Holland Biomedical Press, 1978, pp. 69-73.

In press:

Chesebro, B.: The influence of the major histocompatibility complex (H-2) on oncornavirus-induced neoplasia in mice. In Kaiser, H. E. (Ed.): Comparative Pathology of Abnormal Growth

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00075-03 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunology of Pathogenic Fungi

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. F. Hasenclever

OTHER: None

COOPERATING UNITS (if any) None

LAB/BRANCH

Rocky Mountain Laboratory, Hamilton, MT 59840

SECTION

Allergy and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00076-20 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immunopotentiation by Components of Microbes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. A. McLaughlin	Senior Staff Fellow	RML NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	RML NIAID
	J. L. Cantrell	Senior Staff Fellow	RML NIAID
	K. B. Von Eschen	Staff Fellow	RML NIAID
	K. Fukushi	Visiting Scientist	RML NIAID

COOPERATING UNITS (if any) Dr. M. T. Kelly, VA Hospital, Salt Lake City, UT; Dr. E. P. Goldberg, Dept. Engineering, Univ. of Florida, Gainesville; Dr. C. H. Granatek, M.D. Anderson Tumor Institute, Houston, TX.

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to delineate, at the cellular and molecular level, the mechanism of action of microbial components in immunopotentiation. In the past year, four major efforts have been made: 1) elucidating the nature of biological responses to treatment of tumors with mycobacterial cell wall derivatives combined with endotoxic extracts obtained from Salmonella typhimurium, 2) investigating the minimal structural requirements of cell wall derivatives responsible for their antitumor activity when combined with endotoxic extracts, 3) quantitative determination of the molecular interaction of these microbial substances with each other and with oil droplets of oil-in-water emulsions used as therapeutic vehicles, and 4) determining the nature of interaction of radiolabeled endotoxin with tumor cells, in vitro, by quantitative binding studies and electron microscopic autoradiography.

PHS-6040
(Rev. 10-76)

Project Description:

In general, our major objective is elucidating the structural requirements and mechanism of action of immunopotentiating components isolated from microbes.

Previously we had shown that a mixture of extracts isolated from mycobacteria and rough mutants of Salmonella typhimurium were more effective in regressing tumors than any of these materials alone. We asked whether this effect was synergistic or additive. Synergy would be characteristic of a phenomenon whereby the microbial components acted independently to stimulate immunological responses in separate but cooperating pathways or steps that culminated in potent antitumor effects. One mechanism for an additive effect would be a single immunological event that was influenced by the different microbial substances. Extensive dose-response studies revealed that killed mycobacterial whole cells, cell walls, deproteinized cell walls, or cell wall skeleton acted synergistically with endotoxic extracts. Animals cured of tumors treated with these preparations developed systemic tumor immunity which suggested that the bacterial products may have acted, at least in part, as classical adjuvants to stimulate immunity to tumor-associated antigens. This antitumor effect was dependent upon delivering the bacterial components in oil-in-water emulsions injected directly into the dermal tumors.

We studied the nature of the requirement for oil-in-water emulsions by developing quantitative assays to determine the amount of mycobacterial or endotoxic substance that associated with oil droplets of oil-in-water emulsions. One assay utilized chemical analyses of the nitrogen content of the oil and aqueous phases separated by centrifugation of the emulsions. The other assay involved lactoperoxide-catalyzed iodination of amino acid residues of proteins in mycobacterial cell wall skeleton. The percentage of cell wall preparation in the oil and aqueous phases of the emulsions could then be quantified by using a gamma counter. A positive correlation was found between the ability of mycobacterial cell wall preparations to bind to oil droplets of oil-in-water emulsions and the antitumor activity of these preparations. In addition, we found that treatment of cell wall skeleton with trehalose dimycolate enhanced the interaction of cell wall skeleton with oil droplets and augmented its antitumor activity. This treatment was thought to cause a noncovalent lipidation of cell wall skeleton which is depleted of free lipids. We concluded that the binding of mycobacterial cell wall preparations to oil droplets is driven by hydrophobic interaction of oil droplets with the lipid-rich portions of the cell wall preparations. Cell wall skeleton was envisioned as interacting with oil droplets via covalently-linked mycolic acid residues as well as via trehalose dimycolate when it was used as an agent to noncovalently lipidate the cell wall skeleton. Studies are in progress to identify the immunological mechanism(s) involved in enhanced immunopotentiating activity induced by lipidation of cell wall skeleton with trehalose dimycolate. Our working hypothesis is that complexes of cell wall skeleton, trehalose

dimycolate, and oil droplets are biologically active depots that slowly release cell wall skeleton to produce a prolonged stimulus locally and systemically in the tumor-bearing host. In addition, or alternatively, these biological active complexes may be processed preferentially by macrophages, thereby augmenting the immunological properties of cell wall skeleton that are important in tumor regression.

Synergistic antitumor activity of cell wall skeleton combined with endotoxin was also found for oil-in-water emulsions wherein endotoxin was entirely solubilized in the aqueous phase, provided that cell wall skeleton interacted with the oil droplets. If both the endotoxin and cell wall skeleton were in the aqueous phase, treated tumors did not regress. Since endotoxin in aqueous solution causes an early hemorrhagic necrosis of treated tumors, we speculate that the synergistic effect of combining endotoxin with cell wall skeleton was, in part, due to an early, host-mediated, cytoreductive effect induced by endotoxin. We speculate that the reduced tumor cell burden could then be more easily destroyed by immunological processes stimulated by the more slowly acting cell wall skeleton.

Last year, we reported a similar synergistic, antitumor response to chemotherapy with mitomycin C added to immunotherapy with BCG cell wall preparations. Rapid reduction in tumor size followed injection of mitomycin C directly into tumors. Mitomycin C inhibited tumor cell metabolism and resulted in tumor cell death as was shown by inhibition of uptake radiolabeled thymidine into DNA by tumor cells treated in vitro with mitomycin C. We have been unsuccessful in attempts to demonstrate similar inhibition of tumor cell metabolism in vitro by endotoxin. Experiments so far conducted indicate that endotoxin does not inhibit protein, RNA, or DNA synthesis in the type of tumor cells utilized in the tumor regression studies described above. We conclude that endotoxin has an early effect on tumor cells that is apparently immunologically mediated, and thus, is unlike the direct, cytotoxic effect of mitomycin.

We did find that endotoxin binds to tumor cells, as determined by quantifying radioactivity associated with tumor cells incubated with radiolabeled endotoxin. Electron microscopic autoradiography revealed that radiolabeled endotoxin, or radiolabeled products derived therefrom, associated with the plasma membrane as well as with intracytoplasmic and intranuclear components of the tumor cells. Additional studies are in progress to complete these preliminary investigations of the interaction of endotoxin with tumor cell substructure.

The future course of this project will be concentrated in three major directions: 1) delineating the minimal structural requirements of mycobacterial cell wall skeleton responsible for the synergistic antitumor effect of combining cell wall skeleton with endotoxin, 2) fractionating and characterizing the active components in aqueous extracts of wild-type and

Re mutant S. typhimurium that are responsible for this antitumor activity, 3) elucidating the mechanism of the synergistic activity of cell wall skeleton or chemically synthesized substitutes, when these materials are combined with endotoxin. Studies in progress indicate that one of the substances in mycobacterial cell wall derivatives that may be responsible for this synergy is the adjuvant peptide, N-acetyl-muramyl-alanyl-isoglutamine. Hopefully, we shall be able to synthesize analogs of the adjuvant peptide to ascertain whether other substitutions in the adjuvant peptide structure might heighten its antitumor activity when combined with endotoxin. The use of these synthetic substances should simplify the task of determining the critical mechanisms responsible for the striking biological responses to them. Conceivably, isolation of the active principle or principles in the endotoxic extracts will assist us in determining the critical functions of endotoxic components when combined with mycobacterial cell wall derivatives. Minimizing the number of microbial components will enable us to ascertain with more certainty and precision the mode of synergistic, immunopotentiating activity of these substances at the cellular and molecular level.

Publications:

McLaughlin, C. A., Cantrell, J. L., Ribi, E., and Goldberg, E. P.: Intratumor chemoimmunotherapy with mitomycin C and components from mycobacteria in regression of line 10 tumors in guinea pigs. Cancer Res. 38: 1311-1316, 1978.

McLaughlin, C. A., Parker, R., Toubiana, R., and Ribi, E.: Moieties of mycobacterial mycolates required for inducing granulomatous reactions. Cell. Immunol. 38: 14-24, 1978.

McLaughlin, C. A., Strain, S. M., Bickel, W. D., Goren, M. B., Azuma, I., Milner, K., Cantrell, J. L., and Ribi, E.: Regression of line-10 hepatocellular carcinomas following treatment with water-soluble, microbial extracts combined with trehalose or arabinose mycolates. Cancer Immunol. Immunother. 4: 61-68, 1978.

In press:

McLaughlin, C. A., Ribi, E. E., Goren, M. B., and Toubiana, R.: Tumor regression induced by defined microbial components in an oil-in-water emulsion is mediated through their binding to oil droplets. Cancer Immunol. Immunother.

McLaughlin, C. A., Bickel, W. D., Kyle, J. S., and Ribi, E.: Synergistic tumor regressive activity observed following treatment of line-10 hepatocellular carcinomas with deproteinized BCG cell walls and mutant Salmonella typhimurium glycolipid. Cancer Immunol. Immunother.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00077-22 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Structure and Biological Activity of Endotoxins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. B. Von Eschen	Staff Fellow	RML NIAID
	E. E. Ribi	Res. Chemist (Phys. Chem.)	RML NIAID
OTHER:	C. A. McLaughlin	Senior Staff Fellow	RML NIAID
	J. L. Cantrell	Senior Staff Fellow	RML NIAID

COOPERATING UNITS (if any) Dr. R. Parker and S. M. Strain, Hamilton Biochemical Res. Lab., Hamilton, MT; Dr. J. A. Rudbach and E. Milner, Univ. of Montana, Missoula; Drs. P. Minden and M. Brunda, National Jewish Hospital, Denver, CO; Dr. B. Sharma, Childrens Hosp., Denver, CO; Dr. G. Vosika, Univ. of Minn., Minneapolis.

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Molecular Biology Section

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NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 6/12	1 4/12	1 2/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main objective of this project is to determine the minimal chemical and structural properties of bacterial endotoxins necessary to elicit several biologic and immunologic responses. The purpose is to identify, by fractionation of different endotoxins and chemical, physical, and biological analyses of the individual fractions, the component(s) of the macromolecular complex responsible for the antitumor effects of some endotoxins. Techniques are being developed to assess the cellular nature of the immunomodulatory activities of different endotoxic materials.

PHS-6040
(Rev. 10-76)

Project Description:

We previously reported that certain endotoxic extracts from Gram-negative bacteria, in combination with trehalose dimycolate from Mycobacterium species, regressed established tumors of guinea pigs. This finding has restimulated an interest in our laboratory to study the complex relationship between the structure and biological functions of bacterial endotoxins.

As a continuation of work reported last year, we have evaluated further the relationship between toxicity and antitumor efficacy of certain bacterial extracts. In this context, experiments were done to determine if the antitumor effect of bacterial endotoxin could be attributed solely to the general toxicity of this molecule. Two observations argued against this concept. First, earlier experiments showed that lipopolysaccharides (LPS) extracted from wild type Gram-negative bacteria were highly toxic but relatively ineffective in regressing line-10 hepatocarcinoma tumors in Strain 2 guinea pigs. On the other hand, extracts from Re mutant bacteria (ReGL) were very effective (up to 100% cures) in regressing line-10 tumors and were routinely 10 times less toxic than the wild type LPS. Therefore, there was no direct correlation between relative toxicity and the ability to regress tumors. Secondly, treatment of ReGL with Triton X-100, a nonionic detergent, resulted in a product that was unchanged in toxic, pyrogenic, and mitogenic activities. However, Triton-treated ReGL did not regress line-10 tumors. This latter observation suggested that some component of ReGL, altered or destroyed by exposure to Triton X-100, was necessary for regression of line-10 tumors. Furthermore, both observations indicated that toxicity alone was not sufficient for optimal regression of these guinea pig tumors.

Although there was no direct correlation between toxicity and antitumor effectiveness *per se*, more recent experiments showed that some chemical modifications of ReGL which severely reduced the toxicity of these materials also resulted in a concomitant loss of antitumor activity. More specifically, base hydrolysis, succinylation, phthalylation, and conjugation with polymyxin B markedly reduced the toxicity, pyrogenicity, and mitogenicity of ReGL. Also, electron microscopic analysis revealed that conjugation of ReGL with polymyxin B drastically altered the gross morphological structure of this endotoxic extract. These same modified preparations were relatively ineffective in regressing line-10 tumor whereas unmodified ReGL regressed 70 to 100% of the tumors. Therefore, it was suggested that optimal tumor regression by ReGL required a certain level of toxicity.

Results of experiments using native and chemically modified ReGL led to the following hypothesis: Two functionally distinct portions of the ReGL molecule are required for regression of line-10 tumors and the subsequent establishment of systemic immunity. One portion is related to

the toxic activity of ReGL and the other portion, functionally absent in wild type LPS and Triton treated ReGL, is currently undefined. As a partial test of this hypothesis, experiments using individual or combinations of endotoxic extracts were done. We found that wild type LPS and polymyxin B treated ReGL regressed only low percentages of line-10 tumors (13% for each) when injected separately. However, when these two ineffective reagents were mixed together, 87% of tumor bearing guinea pigs treated with the mixture were cured. Other experiments using combinations of toxic and nontoxic bacterial extracts are in progress and should provide new information on the dose requirements for optimal regression of animal tumors. Such information becomes very important when considering the potential role of bacterial endotoxin as a therapeutic agent for human neoplastic diseases.

To further define the relationship between the structure and function of bacterial endotoxin, we attempted to isolate and characterize fractions from endotoxic extracts. To date the most rewarding studies have been done with an organic solvent extract of an Re mutant of Salmonella typhimurium. This endotoxic glycolipid (termed MCP), extracted according to the method of Nowotny, was soluble in chloroform-methanol, 4:1 and displayed 4 bands following thin-layer silica gel chromatography. On a preparative scale, Dr. Reno Parker of the Hamilton Biochemical Research Laboratory, isolated 3 distinct fractions (B_1, B_2, and B_4) from the starting MCP. One of the fractions, B_4, was highly toxic and pyrogenic but, similar to Triton treated ReGL, only marginally effective in regressing line-10 tumors. Potent antitumor activity was restored when B_4 was combined with certain nontoxic, amino acid containing side fractions (CMI and ACP) which were isolated during the preparation of MCP. Repeated precipitation of B_4 with calcium ions appeared to remove an ingredient needed to obtain cell-mediated antitumor immunity. Interestingly, calcium precipitates of endotoxic ReGL were highly toxic and soluble in chloroform-methanol. Furthermore, calcium precipitates were rendered soluble in water again by removing the calcium ions with EDTA and dialysis against water. Finally, the complex nature of calcium precipitated B_4 was shown as it was chromatographically resolved into at least 15 bands with the aid of an ammonia-containing solvent system. This last observation was interesting because the original starting material, MCP, was resolved into only 4 bands using the same chromatographic system.

Gel diffusion analyses revealed that two distinct antigens were demonstrable when MCP was reacted against rabbit antisera to whole cells of S. typhimurium. One of these antigens was also found in B_4 whereas B_2 contained the other, slower migrating antigen.

The starting MCP, B_1, B_2, B_4 and another extraction product termed ACP were also tested further for mitogenicity for spleen cells from mice genetically responsive or unresponsive to endotoxic mitogens (see below). It was of interest that all preparations were mitogenic for the LPS

responder strains of mice, and only the MCP and ACP were mitogenic for the LPS nonresponder strains. Apparently these latter two preparations contain a material which is distinct from classical endotoxin in terms of mitogenic activity.

Additional experiments were done to describe and characterize a new strain of mouse genetically unresponsive to many of the biological effects of bacterial endotoxins. Results of a standard mitogen assay showed that spleen cells from C57BL/10ScN mice (B10), of the RML colony, were unresponsive to a variety of endotoxic preparations, including ReGL. Spleen cells from B-10 mice made typical mitogenic responses to other T and B cell mitogens and unresponsiveness was not overcome by using different doses of endotoxin or by changing the day of assay. Also, unresponsiveness was not the result of a nonspecific toxic effect of endotoxin on cultured B-10 lymphocytes. Furthermore, B-10 mice were found to be markedly resistant to the lethal effects of endotoxin in vivo and gave aberrant antibody responses following immunization with wild type LPS. Additional studies showed that lymphocytes from B-10 and endotoxin responder mice bound similar amounts of radiolabeled endotoxin. Apparently, B-10 lymphocytes can bind endotoxin but this binding does not result in typical activation of lymphocyte processes. These results were reported at the ICN-UCLA Symposium on Transmembrane Signaling, Keystone, Colorado, February 27 March 1, 1978.

Some experiments were done to determine genetic characteristics of the unresponsive state of B-10 mice. Initial results using offspring from B-10 mice mated with C57BL/6 animals (endotoxin responders) indicated that endotoxin unresponsiveness was an autosomal recessive trait. Currently, we are mating these F_1 mice with B-10 animals so that backcross analyses can be done. Also, F_1 mice from B-10 X C3H/HeJ (another endotoxin unresponsive strain) were found to be unresponsive, suggesting the possibility that both unresponsive strains suffer from the same genetic defect.

Genetic responder and nonresponder mice were also used in studies concerned with the antitumor effects of endotoxic extracts. Preliminary experiments showed that ReGL admixed with viable tumor cells suppressed the growth of tumors when the mixture was injected into endotoxin responder mice. ReGL did not suppress the development of tumors when the mixture was injected into congenic mice which were unresponsive to endotoxin. Other experiments with responder and nonresponder strains of mice were done in conjunction with Dr. J. Cantrell of the Molecular Biology Section.

The future course of this project is to continue our studies on the relationship between the structure and function of bacterial endotoxin, with special emphasis on the requirements for endotoxic extracts to be effective antitumor reagents. We will continue to fractionate endotoxins and analyze individual and combinations of fractions for structural

properties and biological activities. One goal is to isolate on a preparative scale some or all of the 15 moieties which comprise endotoxic B_4. These fractions will then be tested for physicochemical and biological properties.

In addition, we will expand our use of genetic responder and nonresponder mice to include two different congenic pairs of mice (C3H/HeJ and C3HeB/FeJ, C57BL/10ScN and C57BL/6J) and at least two different types of tumors. These test systems will be used to evaluate the antitumor activity of different endotoxic extracts, including materials such as ACP and endotoxin protein which can activate lymphocytes from both the responder and nonresponder strains. Furthermore, we are currently developing methods for the in vitro generation of cytotoxic lymphocytes reactive against syngeneic and allogeneic tumor cells. Since it was reported that endotoxin enhanced the formation of these cytotoxic cells, we hope to use this test system and isolated lymphoid cell subpopulations from endotoxin responder and nonresponder mice to determine which cell types are involved in the immunopotentiating activity of endotoxin. Therefore, we hope to provide more information on the structure-function relationships of bacterial endotoxins as well as a description of the lymphoid cell types involved in the antitumor activity of these bacterial extracts.

Also within the scope of this project, several collaborative projects were initiated and will be pursued in the future. In conjunction with Drs. P. Minden, M. Brunda, and B. Sharma, Denver, antigenic cross-reactivity between endotoxic extracts, BCG, and line-10 tumor cell antigens is being evaluated. Also with these same collaborators, we are studying the capacity of different endotoxic materials to modulate the in vitro generation of cytotoxic lymphocytes reactive against line-10 tumor cells. In conjunction with Dr. G. Vosika, University of Minnesota, the capacity of different endotoxic extracts to activate mitosis in human lymphocytes is being tested. With the help of Dr. J. Rudbach and E. Milner, University of Montana, different test systems are being used to examine the capacity of endotoxin to influence T cell dependent immune responses. Finally, Dr. R. Wheat, Duke University Medical Center is isolating and purifying proteins from the outer membranes of Re mutant bacteria. Like our endotoxic extracts, these proteins will be tested for chemical, structural, and biological properties. Also, the contribution of these outer membrane proteins to the biological activities of ReGL will be analyzed.

Publications: None

Appendix 1. Contract N01 AI 72525. Production of P3 and Isolation, Fractionation, and Purification of Lipopolysaccharides (Endotoxins), Hamilton Biochemical Research Laboratory, Hamilton, Montana.

Total Man Years: 3 Professional: 8/12 Other: 2 4/12

Annual Funding: $55,000

This project was designed, in part, to study and develop methods for the isolation, fractionation, and purification of bacterial endotoxins. To date the contractor has used different solvent systems to move endotoxins through silica gels during centrifugal chromatography. In preliminary experiments, he has separated endotoxin into 3 fractions, of which 2 were nontoxic and inactive in tumor regression.

The third fraction was toxic and when combined with other products extracted from Gram-negative bacteria regressed tumors. Also, preliminary evidence suggested that this toxic fraction consisted of 15 different substances, and attempts are now being made to isolate these different substances on a preparative scale.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00078-06 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Microbial Components in Cancer Immunotherapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	C. A. McLaughlin	Senior Staff Fellow	RML NIAID
	J. L. Cantrell	Senior Staff Fellow	RML NIAID
	K. B. Von Eschen	Staff Fellow	RML NIAID

COOPERATING UNITS (if any) Dr. B. Zbar, NCI; Dr. W. Brehmer, Robert Koch Institute, Berlin; Drs. S. Richman, C. H. Granatek, and K. Hwang, M.D. Anderson Hospital, Houston, TX; Dr. G. Vosika, University of Minnesota, Minneapolis; Dr. R. Parker and S. M. Strain, Hamilton Biochemical Research Laboratory, Hamilton, MT.

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	2 1/12	3 11/12

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of this project is to identify nonviable, chemically defined microbial components that are efficacious antitumor agents and to develop and supply such preparations in stable standardized forms for both experimental and clinically applied immunotherapy. The intent of the research is to circumvent the use of infectious agents, and by preparing synthetic analogues, provide alternative substances that are not antagonistic or that do not produce undesirable biological responses.

PHS-6040
(Rev. 10-76)

Project Description:

The empirical goal to develop clinically acceptable immunostimulants for tumor regression has remained a major part of the research efforts of the Molecular Biology Section. As is evident from the annual reports submitted individually by Drs. McLaughlin, Cantrell, and Von Eschen, its members collaborate in a multilevel approach to elucidate the mechanism of immunomodulation by microbial components.

Local lesional immunotherapy in a guinea pig has been used as a model approach to treatment of cancer in man. Viable Bacillus Calmette-Guérin (BCG) or formalinized whole cells of the Q fever rickettsia or Corynebacterium parvum were effective in treating a carcinogen-induced hepatocarcinoma (line-10) implanted into the skin of syngeneic guinea pigs. Similar results could be obtained with refined bacterial components, such as BCG cell walls associated with oil droplets in saline suspension. By fractionating the BCG cell wall, we have prepared components that retain antitumor activity and have the potential advantage of reduced allergenicity. A combination of two components in an oil droplet vaccine was essential. One compound is the cell wall skeleton (CWS) which is an insoluble polymeric mycolic acid-arabinogalactan-mucopeptide that is virtually free of tuberculoprotein. The other component is a chloroform-soluble fraction of the free mycobacterial lipid (P3) which is a mixture of trehalose diesters of 88-carbon mycolic acids. In last year's report, we have shown that the nonantigenic portion of our adjuvant, P3, can be replaced by a synthetic analogue of simpler structure and lower molecular weight (trehalose diester of 44-carbon behenylbehenic acid).

We report here that the antigenic adjuvant-active portion of CWS can also be replaced by a simple synthetic analogue resembling a fragment of the mucopeptide moiety of CWS. The mucopeptide moiety partially consists of subunits containing N-acetyl-glucosamine-N-acetyl muramic acid and a series of peptide-linked amino acids in the sequence of L-alanine, D-glutamic acid, and M-diaminopimelic acid. The smallest adjuvant-active unit of the CWS structure, proven by synthesis by French and Japanese workers, appears to consist of N-acetyl muramyl-L-D-isoglutamine. However, muramyl dipeptide in which alanine was replaced by serine (prepared by S. M. Strain under an NIAID contract with Hamilton Biochemical Research Laboratory) when combined with P3, provided line-10 tumor regressive potency superior to that of the alanine-containing product. The muramyl dipeptides and muramyl dipeptides lipidated by covalently linking the muramic acid residue with mycolic acid were not active. Also, when serine was replaced by glycine, the tumor regressive activity of the muramyl dipeptide was completely abolished. The specificity of the antigenic alanine- or serine-isoglutamine portion may play an important role in the mode of action of bacterial adjuvants because of probable prior exposure of man and animals to bacteria carrying these determinants. Therefore, microbial adjuvants may not act as nonspecifically as generally presumed,

but rather by virtue of an immunological T-cell response to the antigenic portion of the adjuvants. Such a response may cause the release of the soluble mediators which stimulate effector cells such as tumor-killing macrophages.

We found that P3 can be combined with any of several microbial immunogens or antigens to form therapeutic agents, some of which are far more effective than BCG cell walls. The most potent antitumor adjuvants are phenol-water or chloroform-methanol extracts from Salmonella typhimurium and S. minnesota Re mutants. These extracts contain endotoxic glycolipids, designated here as Re glycolipids. The synergistic antitumor effects of Re glycolipid, when combined with P3 and/or CWS, have been described in Project No. Z01 AI 00076-20 (McLaughlin). Dr. Reno Parker of the Hamilton Biochemical Research Laboratory fractionated the Re glycolipid by means of microparticulate pressure elution chromatography and obtained a potent endotoxin designated B4, which was essentially devoid of tumor regressive potency, the loss of which was paralleled by the removal of amino acid-containing contaminants from the original Re glycolipid. Indeed, combining B4 with certain unidentified nontoxic side fractions obtained during the isolation of the original Re glycolipid resulted in restored tumor immunotherapeutic potency. Studies are in progress to determine whether these materials are fragments (precursor or degradation products) of the gram-negative bacterial cell wall that are structurally similar to or identical with those present in the BCG CWS. These unidentified components may contain the antigen that cross-reacted immunologically with BCG CWS, Re glycolipid, and line-10 tumor cells as observed by Dr. Granatek. Work is in progress to isolate and identify the cross-reactive component(s). Dr. Percy Minden of the National Jewish Hospital in Denver, a co-discoverer of antigen(s) shared by BCG, other bacteria, line-10 tumor cells, and certain human tumors, such as malignant melanoma, has also offered his collaboration in this venture. One of our working hypotheses is that cross-reactive antigens shared by microorganisms and tumor cells represent immune determinants against which P3, suitable synthetic substitutes of P3, or Re glycolipid augment antitumor immunity.

An unanswered question is why do bacterial immunostimulants cause host-mediated destruction of tumor cells rather than of normal cells? One possibility is that eukaryotic, cell-associated antigens that might cross-react with those of microorganisms may be cryptic in normal cells, but those of tumor cells are exposed on the surface. A similar analogy may be drawn for polysaccharide-rich endotoxic extracts (analogous to normal cells) from wild-type gram-negative bacteria versus Re glycolipid-rich extracts (analogous to tumor cells) from polysaccharide-deficient mutant organisms. Following treatment of lipopolysaccharides with mineral acid, the residual material, in contrast to the original complete endotoxin, cross-reacted serologically with tumoricidal Re glycolipid. In other words, the wild-type bacteria obviously contain the same antigen as the mutants but it is not available to participate in tumoricidal activity until it is exposed as a result of hydrolysis.

Guinea pigs and mice tolerate doses of Re glycolipid required to regress line-10 tumors and an Investigational New Drug Permit for Re glycolipid plus P3 or Re glycolipid plus CWS plus P3 has been obtained for Phase I trials in patients at M.D. Anderson Hospital in Houston. However, we are continuing to explore the possibility of reducing the toxicity of Re glycolipid by chemical and physicochemical modifications and by preparing synthetic analogues of low toxicity. Ideally, we would hope to prepare a nontoxic Re glycolipid with tumor-regressive properties. Studies to date indicate that at least a certain level of toxicity may be necessary for optimal antitumor activity.

Over the past 2 years, Dr. Vosika investigated in Phase I trials the efficacy of intralesional immunotherapy utilizing CWS plus P3 attached to oil droplets. Fifteen patients with metastatic malignant melanoma having cutaneous and/or lymphatic disease with or without visceral disease and a life expectancy of about 2 months were treated with this material. Patients received 300 µg to 1050 µg of the CWS combined with one-half that amount of P3 every 1 to 2 weeks for a total of 8 treatments. Therapy was continued if regression of injected lesions only occurred, but it was discontinued if regression of noninjected disease also occurred. As reported last year, CWS-P3 combinations were essentially nontoxic; the major manifestation was fever up to 101° F that usually subsided within 24 hours.

Six of the 15 patients had regression of at least one injected lesion. Four of these 6 patients also had regression of noninjected disease lasting 4+, 6, 16, and 18+ months. Six of 7 patients who reacted to one of a battery of skin tests responded to therapy whereas all 8 patients who did not react to skin tests failed to respond to therapy.

These data agree with and support the concepts formed during previous studies using viable BCG for intralesional immunotherapy that successful intralesional immunity in malignant melanoma requires an intact cellular immune system. Immunotherapy has been thought of as primarily an approach to those patients with either minimal residual disease, or those clinically disease-free but with a high risk for recurrence. In the current study, patients with relatively large tumor burdens, i.e., visceral disease, responded if their immune systems were intact. These results, therefore, alter our concepts of immunotherapy in demonstrating that immunotherapy is an effective single modality in patients with large tumor burdens.

Although the overall response rates to intralesional CWS plus P3 is modest, the response rate increases to 83% (6 of 7) for injected lesions and to 57% (4 of 7) for systemic disease when only skin test-reactive patients are considered. This has prompted us to use CWS-P3 therapy as the primary agent in the treatment of patients with recurrent cutaneous melanoma and to investigate its use parenterally in patients with recurrent noncutaneous malignant melanoma.

The present data show responses in patients with visceral disease which, according to the literature, are not equalled by the use of viable BCG. Also, because the use of killed products eliminates any potential for systemic BCG disease, we are further encouraged to prepare chemically-defined agents and to conduct clinical studies using these pure immuno-adjuvants.

Dr. Stephen Richman, M.D. Anderson Hospital, Houston, extended his clinical trials to include a few patients with cutaneous or subcutaneous breast carcinoma which had recurred after primary surgery. Nodules of 3 of 5 patients were resolved following intralesional injection of CWS plus P3. Intracavitary administration of these materials into patients with advanced refractory carcinoma of the breast resulted in a change from malignant to nonmalignant cytology in 3 of 5 patients, and in the fourth patient the effusion changed to degenerating tumor cells. The effusion volume decreased in 2 patients and stabilized in another. Toxic manifestations were not severe enough in any case to require cessation of therapy. These encouraging results must be considered preliminary.

Several major courses of action are planned in this project: 1) Synthetic work to be continued by preparing muramyl peptides containing various combinations of amino acids. The most effective of these monomeric combinations will then be cross-linked to form oligomers or polymers whose structures more closely resemble that of naturally occurring CWS. We suspect that such compounds, which would be less soluble than the monomeric muramyl peptides, will initiate cell-mediated immunity that is slower to progress but longer lasting and thus minimize adverse immediate physiological reactions for which low molecular weight synthetic products are noted. 2) Continue the preparation and testing of synthetic products which might favorably replace P3. 3) Continue collaboration with Drs. Granatek and Minden in attempts to isolate and identify the structure of cross-reactive components shared by microorganisms and neoplasms, including line-10 hepatocarcinoma and human malignant melanoma. These studies are in addition to the ongoing collaboration with Dr. Springer (see Project No. Z01 AI 00087-01 (Cantrell) which deals with the recognition of blood group antigens which immunologically cross-react between microorganisms and neoplasms. 4) Continue collaboration with Drs. Vosika and Richman.

Publications:

Strain, S. M., Toubiana, R., Ribi, E., and Parker, R.: Separation of the mixture of trehalose 6, 6'-dimycolates comprising the mycobacterial glycolipid fraction, "P3". Biochem. Biophys. Res. Commun. 77: 449-456, 1977.

Publications: (cont'd)

Toubiana, R., Ribi, E., McLaughlin, C., and Strain, S. M.: The effect of synthetic and naturally occurring trehalose fatty acid esters in tumor regression. Cancer Immunol. Immunother. 2: 189-193, 1977.

Zbar, B., Ribi, E., and Rapp, H. J.: Immunotherapy of a guinea pig hepatoma with living BCG. A frozen liquid and a lyophilized preparation are equally active. Cancer 40: 2930-2932, 1977.

Ribi, E., Toubiana, R., Strain, S. M., Milner, K. C., McLaughlin, C., Cantrell, J., Azuma, I., Das, B. C., and Parker, R.: Further studies on the structural requirements of agents for immunotherapy of the guinea pig line-10 tumor. Cancer Immunol. Immunother. 3: 171-177, 1978.

Hunter, J. T., Meltzer, M. S., Ribi, E., Fidler, I. J., Hanna, M. G., Zbar, B., and Rapp, H. J.: Glucan: attempts to demonstrate therapeutic activity against five syngeneic tumors in guinea pigs and mice. J. Natl. Cancer Inst. 60: 419-424, 1978.

Kelly, M. T., McLaughlin, C. A., and Ribi, E. E.: Eradication of microscopic lymph node metastases of the guinea pig line-10 tumor after intradermal injection of endotoxin plus mycobacterial components. Cancer Immunol. Immunother. 4: 29-32, 1978.

In press:

Vosika, G. J., Schmidtke, J. R., Goldman, A., Ribi, E., Parker, R., and Gray, G. R.: Intralesional immunotherapy of malignant melanoma with Mycobacterium smegmatis cell wall skeleton combined with trehalose dimycolate (P3). N. Engl. J. Med.

Richman, S. P., Gutterman, J. U., Hersh, E. M., and Ribi, E.: Phase I-II study of intratumor therapy with BCG cell wall skeleton plus P3: brief communication. J. Natl. Cancer Inst.

Wepsic, H. T., Tracey, R. S., Harris, S., Ribi, E., and Morris, H.: The effect of Bacillus Calmette-Guérin cell walls upon the metastatic potential of transplantable Morris hepatomas. Cancer Res.

Ribi, E., McLaughlin, C. A., Cantrell, J. L., Brehmer, W., Azuma, I., Yamamura, Y., Strain, S. M., and Hwang, K. M.: Immunotherapy of tumors with microbial constituents or their synthetic analogues. A review. Immunotherapy of Human Cancer

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00079-19 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
The Encephalitides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. A. Thomas	Res. Microbiologist	RML NIAID
OTHER:	J. E. Coe	Medical Officer	RML NIAID

COOPERATING UNITS (if any)
Dr. Martin Skinner, Montana State Epidemiologist, Helena, MT.

LAB/BRANCH
Rocky Mountain Laboratory, Hamilton, MT 59840

SECTION
Epidemiology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2/12	1/12	1/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Since we have shown garter snakes capable of developing antibody following experimental infection of Western equine encephalitis, as measured by a modified neutralization test, the study to determine the duration of antibody response has been continued. Attempts are made to establish the identification and serological relationship of field viral isolates from ticks and mosquitoes using a variety of techniques such as hemagglutination, hemagglutination-inhibition, complement-fixation, neutralization, and fluorescent-antibody. The laboratory has continued to assist in the surveillance of encephalitis viruses and certain rickettsial disease agents in Montana and in other states when requested by providing virus isolation and serodiagnostic services to physicians and veterinarians.

PHS-6040
(Rev. 10-76)

Project Description:

A long-term study of antibody response in garter snakes experimentally infected with Western equine encephalitis (WEE) virus, employing a modified regular neutralization test and the snake globulin precipitation test, has been continued to add to our knowledge of the possible role these cold-blooded vertebrates might play in the ecology of WEE. Antibodies were present in 2 of 3 snakes bled 1,366 days (7-26-77) following the initial inoculation and a second inoculation on day 74. Quite a number of snakes have died in recent months and because of this mortality all remaining snakes were sacrificed on 6/2/78 (1,676 days after initial inoculation of WEE virus). Sera have been stored at -20°C to be tested for presence of antibody.

Surveillance of WEE, St. Louis encephalitis, and California encephalitis virus activity to gain more knowledge regarding the ecology of these viruses has been continued. Of 80 Montana horses tested (paired sera from 11), complement-fixing antibodies of a titer 1:8 or greater were detected in 19 (24%) against WEE. Of 8 humans tested, a significant rise in WEE complement-fixing antibodies was detected in one from Washington, and two each from South Dakota and Wyoming.

The cooperative study with other members of the RML staff on tick-borne virus diseases has been continued. This requires maintenance of a large collection of recognized viruses, antigens, and immune sera with which to compare or identify field isolates. Tests employed in the study are pathogenicity in mice, hemagglutination, hemagglutination-inhibition, and neutralization. The number of virus strains (field isolates) and the serological group to which they belong are as follows: 1 Quaranfil, 1 Soldado, 10 Hughes, and 1 Uukuniemi.

In 1978, the department included in its diagnostic service the fluorescent antibody (FA) test to identify Colorado tick fever (CTF) isolates in mice and antibody against Rocky Mountain spotted fever (RMSF) and typhus fever. CTF virus has been identified in 32 of 74 (44%) patients' blood samples submitted for attempted isolation of CTF virus. FA titers of 1:32 against RMSF have been demonstrated in 54 of 326 (17%) human sera tested. All submitted sera have been tested in the FA test for RMSF and typhus antibodies.

The current project on persistence of antibody in snakes is near completion. Diagnostic service, except for RMSF and CTF, has been discontinued. Study of the tick-borne encephalitis viruses will be continued. Laboratory personnel will be tested annually for possible laboratory-acquired infections. In the immediate future, work on the enzyme-linked immunoassay test will be undertaken. Disease agents proposed for investigation are rabies, CTF, RMSF, tularemia, and possibly others.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00080-11 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Tick-Borne Disease Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. E. Yunker	Scientist Director	RML NIAID
OTHER:	C. M. Clifford	Scientist Director	RML NIAID
	J. E. Keirans	Res. Entomologist (Med.)	RML NIAID
	L. A. Thomas	Res. Microbiologist	RML NIAID

COOPERATING UNITS (if any) Dr. J. Casals, Yale Arbovirus Res. Unit, New Haven, CT; Mr. R. Gresbrink, Oregon State Bd. of Hlth., Portland; Dr. H. Hoogstraal, NAMRU-3 Cairo, Egypt; Dr. B. Easterday, Dept. of Vet. Sci., Univ. of Wisconsin, Madison; F. Murphy, CDC; E. Easton, So. Dak. State Univ.; D. G. McKercher, U. Calif.

LAB/BRANCH

Rocky Mountain Laboratory, Hamilton, MT 59840

SECTION

Medical Zoology and Zoonotic Diseases Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3 5/12	1 1/12	2 4/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In this project the incidence, distribution and ecology of tick-borne disease agents, arboviruses and rickettsiae, are studied. These pathogens are isolated in tissue cultures or animals from field-collected materials and are serologically identified. Viruses, if new, are physicochemically and biologically characterized and taxonomically classified. Of particular importance is the delineation of vector-relationships and human disease potential of the increasing number of tick-borne viruses in the United States.

PHS-6040
(Rev. 10-76)

Project Description:

Viruses and rickettsiae in ixodid ticks. Continuing collaboration with WHO/Univ. Wisconsin influenza investigators has resulted in the recognition of three viruses from seabird parasites, Ixodes uriae, collected on St. Paul Island, Alaska. Two of these were cloned by plaque isolation and characterized by IF, CF and NT tests. One is an Orbivirus of the Kemerovo group (Reoviridae) previously found by us on St. Paul Island in blood of a seabird. It is closely related to and possibly indistinguishable from Okhotskiy virus recently described by Soviet workers from ticks collected on islands and coastal areas of the Sea of Okhotsk. Another is a member of the Uukuniemi group (Bunyaviridae) and is distinct from all other members of the group by every serological test. The third agent represented in these ticks, a Sakhalin group virus (unclassified), could not be isolated but its presence was recognized by CF reactions to inoculated tissue cultures. Results will be prepared for publication.

We continued to test samples of the man-biting Dermacentor variabilis collected in Eastern and Western states of the U.S. for evidence of infection with the human pathogens, Powassan encephalitis, and Colorado tick fever (CTF) viruses. Tests to date are negative, despite the facts that D. variabilis is capable of transmitting these viruses experimentally and some of the areas of collection are endemic for the viruses.

Upon request of state health authorities or investigators in Oregon, Idaho, Washington, and South Dakota, ticks and human blood samples were routinely tested for evidence of infection with tick-borne pathogens. This resulted in the following isolations: 1 CTF from D. andersoni and 10 Rickettsia montana from D. variabilis in South Dakota, 6 R. rhipicephali from D. occidentalis and 6 isolates of a new rickettsia of the Rocky Mountain spotted fever (RMSF) group which we previously described from I. pacificus ticks from Oregon. In addition, a series of D. andersoni taken from black bears in Idaho yielded two isolations of R. rickettsii.

Viruses in argasid ticks. Nine strains of viruses were isolated from Cliff Swallow parasites, Argas cooleyi, taken in South Dakota nesting habitats. Two viruses previously described by us from Texas, New Mexico, and Colorado were recognized in this material: Sapphire II, an unclassified virus of the Hughes serogroup, and Sixgun City, an Orbivirus of the Kemerovo group. A large series of cimicids (bat-bugs) collected from the same habitat were also tested with negative results. (In Texas, these blood-feeding insects had previously been shown to harbor a group A arbovirus closely related to Western equine encephalitis.)

Last year, we reported the isolation of an unidentified virus of the Quaranfil group from South African ticks of the Ornithodoros capensis group. We have now established identity of the virus as Johnston Atoll, first reported by us from Central Pacific Islands and subsequently in

Australia. An undescribed, unclassified virus which we earlier found in A. brumpti from Tanzania has been physicochemically and biologically characterized in preparation for publication. Still lacking from this characterization is electron microscopic (EM) information on morphology and morphogenesis.

Upon request we studied two agents isolated by California workers from cattle and their tick-parasites, O. coriaceus. Both agents were suspected as the cause of epizootic bovine abortion. Neither was identifiable in screen CF tests in which we employed serological reagents of 188 arboviruses. Results of extensive efforts at characterization suggested that one of these was not an arbovirus; subsequently, it was shown by EM to be a mycoplasma (F. Murphy, pers. comm.). The other isolate, still serologically unidentified, possesses viral characteristics and is being further studied.

Assay systems for tick-borne viruses. As previously noted, tick-borne viruses are of low infectivity and antigenicity for mice. All of the viruses mentioned above were isolated in Vero cell cultures but could not be recovered in RML mice. In an effort to determine if strain differences among mice were responsible for failures to isolate these viruses, we compared 4 strains of white mice in their response to i.c. inoculation of serial dilutions of 9 tick viruses. In addition, 24 frozen tick triturates that had previously yielded virus in Vero cells were thawed and inoculated into the different mouse strains as well as in Vero cells. No significant differences were seen among strains of mice in their quantal response to infection.

Cross-testing of 6 Uukuniemi group and 16 Kemerovo group viruses by NT, CF and IF procedures has been completed and will serve as a basis for a proposed working classification of these viruses.

A recently established cell line from the South African clawed frog was obtained from the London School of Hygiene, where it had shown promise in the study of some tick-borne viruses. We tested its ability to form plaques in response to infection with 105 arboviruses and 15 species of Rickettsiales. It is the best system to date for plaque assay of Sakhalin group viruses and shows some advantages over Vero cells in assay of Uukuniemi and Congo-Crimean hemorrhagic fever group viruses as well as spotted fever rickettsiae and certain Wolbachia species. One manuscript describing these results has been completed and another is in preparation.

This project will proceed along lines similar to those of previous years. Collaborative laboratory studies, such as the one on enzootic bovine abortion, serve to broaden the scope of this project. Studies of complex antigenic relationships among tick-borne viruses and development of methods for these studies have implications of benefits in other areas (e.g., vaccine production, diagnosis). Our characterization of new viruses will

be augmented with EM studies on morphology and morphogenesis, technical time permitting. Documentation of the occurrence of tick-borne viruses in various parts of the world, correlating with flyways of migratory birds as well as distributional ranges of tick-vectors, provides predictive information about arbovirus distribution. For example, in 1975 we suggested the presence of Soldado (Hughes serogroup), a Neotropical virus, in the United States. In the following year this prediction was borne out when we isolated that virus from vector ticks collected in Texas. Additional examples are our isolations of Australo-Pacific viruses in both Texas (last report) and South Africa (this report). Nearly all of the viruses noted above cause human illness or are serologically related to human pathogens. The mosquito-borne viruses are relatively well-studied compared to those borne by ticks. This project is singular in its objective to fill an obvious void in the understanding of tick-borne pathogens. Emphasis is placed on work that receives little or no attention elsewhere.

Publications:

In press:

Clifford, C. M.: Tick-borne viruses of seabirds. Proc. 2nd Int. Symp. on Arctic Arboviruses, Mont Gabriel, Que., Canada, May 26-28, 1977.

Yunker, C. E., Thomas, L. A., and Cory, J.: Phenetic relationships of viruses of the Hughes serological group. Proc. 2nd Int. Symp. on Arctic Arboviruses, Mont Gabriel, Que., Canada, May 26-28, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00081-17 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
The Epidemiology of Human Infections of Special Interest

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. N. Philip	Medical Director	RML NIAID
OTHER:	E. A. Casper	Nurse Director	RML NIAID
	L. A. Thomas	Research Microbiologist	RML NIAID
	W. Burgdorfer	Res. Entomologist (Med.)	RML NIAID

COOPERATING UNITS (if any) Dr. J. P. Davis, Duke University Medical Center, Durham, NC; Dr. K. Hechemy, NY State Dept. of Health, Albany, NY.

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SECTION
Epidemiology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 7/12	1	7/12

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☒ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project emphasizes epidemiologic investigations of infectious diseases of particular interest to the research program of the Rocky Mountain Laboratory. Current investigations include a study of Colorado tick fever in the Northwest, the etiologic relationship of various spotted fever-group serotypes to human illness, and improvement in laboratory methods for diagnosis of rickettsial infections, particularly Rocky Mountain spotted fever.

PHS-6040
(Rev. 10-76)

Project Description:

Colorado tick fever (CTF). The epidemiologic study of CTF will be terminated after this season. Sixty-two laboratory-confirmed cases of CTF from 7 states (including 21 from the Bitterroot Valley) were identified in 1977. Thirty-eight cases (including 13 from the Bitterroot Valley) have been diagnosed thus far this year. As in previous years, all cases were mild and without complications. In two instances, infection was acquired in Ravalli County, but illness did not occur until the patients had arrived home in states where CTF does not occur. This has happened on at least three other occasions and indicates some of the problems of diagnosis in a highly mobile society.

CTF and Rocky Mountain spotted fever (RMSF). In collaboration with the Rickettsial Diseases Section, we have continued to screen attached ticks submitted by local residents for CTF virus and spotted fever-group (SFG) rickettsiae. During the 3 years of study, 723 attached ticks have been submitted for examination. Of these, 7.3%, 1.5% and 6.2% contained CTF virus, SFG rickettsiae and SFG-nonstaining bacillary forms, respectively, in the hemocytes. Follow-up clinical and epidemiologic information was obtained from 84% of these persons. Sixty-seven (11%) of the 606 persons contacted had various clinical complaints, usually mild, which could have been tick-associated. Forty-eight persons were bitten by CTF virus-infected ticks; less than half developed illnesses, most of which were confirmed as CTF. Of 11 persons bitten by ticks infected with SFG rickettsiae, only 2 (both confirmed as RMSF) developed illness. The strains isolated from both offending ticks were serotyped as Rickettsia rickettsii. Forty-one persons were bitten by ticks containing nonstaining bacillary forms. Ten of these persons subsequently had variable non-specific complaints.

Sera obtained from 14 persons several weeks after bite by ticks infected with rickettsia-like organisms were tested against representative strains of the 10 SFG serotypes indigenous to the United States using the microimmunofluorescence (micro-IF) test. Only 2 had micro-IF antibodies against one or more of these prototypes. Both had highest titers against R. rickettsii, and both were patients with RMSF that had been bitten by the R. rickettsii-positive ticks mentioned above. Information is still fragmentary, but it now appears that the rickettsia-like organisms present in ticks in this area, other than R. rickettsii, are not often, if ever, a cause of human infection.

Laboratory diagnosis of RMSF. As projected in last year's report, we attempted to devise an enzyme-linked immunoassay (ELISA) test for detection of antibody and particularly, SFG antigen for early diagnosis of RMSF, without great success. The problem apparently has to do with development of satisfactory purified antigens that will bind to the assay plates. These efforts will continue. In the meantime, serial urine and serum specimens from RMSF cases in North Carolina are being held by Duke University collaborators for testing.

The micro-IF test is continuing to be used by Dr. Thomas for serodiagnosis of RMSF. In collaboration with the N.Y. State Health Department, a report on the favorable comparison of this procedure with the standard Weil-Felix test of sera from suspect RMSF cases is being readied for publication. In 1977, 20 cases of RMSF were diagnosed by serological responses, including one acquired in the United States but manifest in Great Britain, thus indicating again that a high level of diagnostic acumen is necessary for clinical diagnosis of tick-borne diseases in areas where they are uncommon.

Another approach to early diagnosis of RMSF which we are investigating has to do with examination of suspect tick-vectors. We have developed a procedure for sequential staining of tick hemolymph with monotypic fluorescein-conjugated antibody. In a survey of Bitterroot Valley ticks, this method was shown to be promising for direct identification of the rickettsial organisms in the tick host. Better conjugates are needed, however. We have attempted to conjugate antibody raised in laboratory mice and to dilute the conjugate beyond the level of cross-reacting to antigens shared by SFG strains circulating in this area. However, the mouse is not an ideal system for preparation of potent conjugates. The value of the procedure is dependent on knowledge of the variety of serotypes to be found in a given area.

No further studies of CTF are planned. We will concentrate on developing laboratory procedures for early diagnosis of RMSF. Assessment of serologic response is not the answer. Even with very sensitive serodiagnostic procedures, antibody response to rickettsial infection cannot be detected before 6 to 7 days after onset of illness. This is much too late for the practicing physician to decide upon specific therapy. We still think that R. rickettsii antigen can be detected earlier by such sensitive techniques as the ELISA test, provided technical difficulties can be overcome. We will continue to explore the possibilities for hemolymph testing of attached ticks by sequential direct immunofluorescence. This procedure offers possibilities both in tick surveys for SFG rickettsiae present in a particular area and also for identifying potential cases of RMSF by detection of R. rickettsii in attached ticks.

Publications:

In press:

Philip, R. N.: Scrub typhus. In Steele, J. H. (Ed.): CRC Handbook of Zoonoses

Philip, R. N.: Typhus group rickettsiae. In Steele, J. H. (Ed.): CRC Handbook of Zoonoses

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00082-17 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Relation of Viruses to the Genesis of Chronic Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. J. Hadlow	Res. Veterinarian (Path.)	RML NIAID
OTHER:	R. E. Race	Veterinary Officer	RML NIAID

COOPERATING UNITS (if any) Dr. S. B. Prusiner, Dept. of Neurology, University of California, San Francisco.

LAB/BRANCH

Rocky Mountain Laboratory, Hamilton, MT 59840

SECTION

Slow Viral Diseases Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12 4/12	2 1/12	10 3/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is primarily concerned with characterizing several naturally occurring slow viral diseases of domestic animals that resemble important, etiologically obscure diseases of man. Two diseases receive most attention: scrapie of sheep and goats and Aleutian disease of mink. Their clinico-pathologic, virologic, and immunologic features are studied to provide an understanding of their pathogenesis and natural history. Information from such studies will help define the unusual host-virus relationships that result in protracted disease in man and animals.

PHS-6040
(Rev. 10-76)

Project Description:

This project is concerned with characterizing the unusual host-virus relationships that give rise to slowly evolving diseases after a long incubation period. The main diseases studied are scrapie of sheep and goats and Aleutian disease of mink. Because the primary objective of the research is the study of the disease process in each infection, most attention is directed to obtaining information on the temporal replication of the causative virus, appearance of antibody, and host cellular responses in relation to the natural history of the disease. Less attention is directed to obtaining information on the physical and chemical properties of the causative viruses.

Scrapie and mink encephalopathy. Several observations made here during the past years indicated that mink encephalopathy virus almost certainly is nothing more than scrapie virus that became somewhat changed with one passage in a foreign host, the mink. New observations on the behavior of scrapie virus and mink encephalopathy virus strengthen this view.

All of 3 pygmy goats inoculated intracerebrally with sheep scrapie virus that had been passed 3 times in mink became affected with scrapie about 16 months later. Of particular interest in these goats was the pronounced spongiform change and astrocytosis that was widespread in the cerebral cortex. These histologic changes are not characteristic of natural scrapie in the goat and sheep or of the experimental disease in these hosts that is caused by mouse-passaged scrapie virus of sheep origin. They are, however, characteristic of the neurologic disease that occurred in goats and sheep inoculated with second passage mink encephalopathy virus. Moreover, these cortical changes became much more severe with two additional passages of mink encephalopathy virus in the goat.

Neuropathologically, then, the disease induced in goats by third mink passage scrapie virus and second mink passage of encephalopathy virus (a comparable passage level if the natural disease in mink is assumed to represent the first passage of scrapie virus in this host) are the same. Thus, three passages (maybe fewer) of wild scrapie virus in mink greatly alter the topographic pattern of the neuropathologic changes it causes in goats. The pattern resembles that of encephalopathy in mink.

Although mink encephalopathy virus was readily detected, by mink inoculation, in the brains of two affected goats, it was not detected in non-neural tissues (mesenteric and prescapular lymph nodes, spleen, ileum, and upper colon) except for a trace in the upper colon of one goat. Such limited non-neural distribution of encephalopathy virus in these goats mimics the pattern in mink inoculated with the same virus. This probably is another reflection of altered biologic properties of presumed scrapie virus incident to passage in an unnatural host.

So far, scrapie virus from 7 of 8 Suffolk sheep and from 2 of 2 naturally infected goats caused a neurologic disease in mink that is essentially identical to mink encephalopathy. By contrast, scrapie virus in brains of 2 Cheviot and one Montadale sheep, breeds that are similar genetically but different from Suffolks, did not cause disease in mink. Also, virus in the brains of a Rambouillet and a Targhee sheep, breeds unrelated to the others, was highly infectious for mink even though it was only moderately infectious for the mouse. Apparently, scrapie virus from different breeds of sheep varies greatly in its pathogenicity for the mink. Moreover, our observations suggest that sometimes the mink is more sensitive than the mouse for detecting scrapie virus. Presumably, if titrations of scrapie virus from sheep were done in both mink and mice, the respective titers would differ significantly. A study to determine whether this is so is in progress.

As mentioned in previous annual reports, passage of scrapie virus in mink greatly alters its ability to cause typical scrapie in the mouse. Apart from clinical differences, the disease that occurs is atypical in that neurohistologic changes are found mainly in the cerebral cortex. Subcortical gray masses, brain stem, and spinal cord are not affected as they are in typical murine scrapie. Passage in mice of brain from such atypically affected mice so far has not resulted in typical scrapie. But the same inoculum caused typical scrapie in all of four mink after the usual incubation period of about 15 months.

These several observations on scrapie virus and mink encephalopathy virus not only emphasize their probable common identity but also emphasize the difficulty that may be encountered in attempts to identify, by animal inoculation, the causal role of scrapie virus in neurologic diseases of man.

In a collaborative study with Dr. Stanley Prusiner, the sedimentation behavior of scrapie virus in homogenates of infected mouse brain and spleen were studied as a prelude to obtaining relatively purified virus for closer examination of its properties. Fractions, identified by enzyme and chemical markers, were obtained by analytical differential centrifugation and assayed for infectivity in mice. Most of the virus sedimented with free polyribosomes. Sedimentation properties of the virus were not appreciably changed by heating the preparation to 80°C for 30 minutes, by sonication, or by treatment with sodium deoxycholate and lysolecithin. These preliminary findings suggest that scrapie virus is a discrete particle separable from cellular membranes and is not an integral part of them as the prevailing view declares.

Aleutian disease (AD). Because the Principal Investigator asked to be relieved of major responsibility for this project, little new work on AD was started during the past year. Most of the effort was devoted to gathering together loose ends of experiments begun several years ago. As

yet, the results of these experiments, including a large one on the response of pastel mink to AD virus, have not been evaluated. Nevertheless, an experiment designed to examine more closely the virologic features of AD virus infection induced by intranasal inoculation was started early in the year, but it was abandoned a few months later as a result of an administrative decision to terminate this project.

Creutzfeldt-Jakob (C-J) disease. To complement the work on scrapie, a pilot study on C-J disease was begun about 3 years ago. Mice, guinea pigs, mink, and goats were inoculated with suspensions of brain from 11 persons affected with this disease. So far, a presumed neurologic disease has been recognized only in a few mice. Whether it is related to the inoculation of human brain is not known. Passage of brain from these suspect mice into other suckling and weaned mice was done recently. The guinea pigs will be observed for another 6 months, and the mink and goats for at least another year before they will be discarded.

Neoplastic disease in mink. Neoplastic disease continues to be an important cause of debility and death in our mink that survive 4 to 10 years. Lymphocytic lymphoma is by far the most common form. Its occurrence in a one-year-old sapphire mink inoculated as a newborn with a cell-free filtrate prepared from tissues of an old pastel affected with spontaneous lymphoma may represent successful transmission of the neoplasm. The spontaneous disease does not occur in young mink.

Dr. Race continued his studies with the transplantable fibrosarcoma mentioned last year. Because only newborn mink, which are available but once a year, are susceptible, such studies can be done only during a brief period each year. Observations made this year indicate that mink of the non-Aleutian genotype (pastels) are less susceptible than those of the Aleutian genotype (sapphires). Reasons for this will be sought now that the dose response has been better defined.

As a result of an administrative decision made early in the year, this project will be discontinued at the end of this fiscal year. Such an abrupt change was not anticipated, so several experiments in progress, including especially the collaborative studies with Dr. Prusiner, will be left unfinished. The small group of laboratory workers who devoted most of their time to this project during the past 16 years will be disbanded. The independent animal unit, which has been so vital to the project, will be turned over to the Animal Department for use by others. Also, the death of Dr. Carl Eklund, early Principal Investigator and later Consultant to this project, would seriously affect the future course of the research. Under the new organization of the Rocky Mountain Laboratory, the Principal Investigator will be left to deal with experimental data, much incomplete, now at hand. Other time will be spent in evaluating a large amount of histologic material accumulated during the long course of this project. Whether this project will ever be reactivated is uncertain.

Publications:

Prusiner, S. B., Hadlow, W. J., Eklund, C. M., and Race, R. E.: Sedimentation properties of the scrapie agent. Proc. Natl. Acad. Sci. USA 74: 4656-4660, 1977.

Hadlow, W. J.: High prevalence of thymoma in the dairy goat. Report of seventeen cases. Vet. Pathol. 15: 153-169, 1978.

In press:

Eklund, C. M. and Hadlow, S. J.: Animal viral diseases as models for central nervous system disease in man. In Vinken, P. J. and Bruyn, G. W. (Eds.): Handbook of Clinical Neurology. Amsterdam, North Holland Publishing Co.

Eklund, C. M. and Hadlow, W. J.: Slow viral diseases. In Steele, J. H. (Ed.): Handbook of Zoonoses. Cleveland, CRC Press, Inc.

Hadlow, W. J.: Myopathy associated with congenital hydrocephalus in Hereford calves. In Ward, B. (Ed.): Spontaneous Animal Models of Human Disease. New York, Academic Press

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00083-10 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Host-Ectoparasite Relationships

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. K. Wikel	Staff Fellow	RML NIAID
OTHER:	J. F. Bell	Medical Director	RML NIAID
	J. E. Coe	Medical Officer	RML NIAID

COOPERATING UNITS (if any) Dr. J. R. Allen, University of Saskatchewan, Saskatoon; Dr. R. O. Drummond, U.S. Livestock Insects Lab., USDA, Kerrville, TX; Dr. W. A Nelson, Agriculture Canada Res. Station, Lethbridge; Dr. R. Osburn, U.S. Livestock Insects Lab., USDA, Kerrville, TX.

LAB/BRANCH

Rocky Mountain Laboratory, Hamilton, MT 59840

SECTION

Medical Zoology and Zoonotic Diseases Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1 2/12	10/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to further characterize the nature of host resistance to ectoparasite infestation, particularly with ticks. Studies examining the nature of resistance to louse infestation have centered on the e of immunosuppressants, athymic mice, and the genetics of the response. Attemp are made to adaptively transfer the resistance response. During the past year evidence has been obtained to show that the alternate pathway of complement activation is important in the resistance response. Histamine levels have bee shown to be elevated at the site of tick attachment on resistant animals; however, studies in which antihistamines with activity against H_1 and H_2 recep tors were used indicate that histamine might not be important in the actual expression of resistance. Hosts resistant to tick infestation were protected against a challenge infestation with ticks infected with highly virulent Francisella tularensis. The role of the Langerhans cell in trapping tick ant gens has also been examined. Studies on immunization against tick infestatio are continuing with marked success. Studies on the immune response of cattle to tick infestation have been initiated.

Project Description:

Blood feeding arthropods are extremely important as vectors of pathogens to man and animals. The suffering and economic losses caused by these ectoparasites and the pathogens that they transmit are vast. Ticks and lice are both important parasites in this respect. The main emphasis in this program has been to examine the immunologic aspects of the tick-host relationship and to a lesser extent the louse-host association. The ultimate goal of both these areas of investigation is to determine the nature of the host response which limits the feeding of the ectoparasite and to utilize this information to develop a means of immunomanipulating the potential host so that the feeding of the arthropod can be reduced or prevented and the subsequent spread of pathogens blocked.

The tick-host association studies have centered around the immunologic aspects of the interaction of the ixodid tick, Dermacentor andersoni, and its hosts, guinea pigs, rabbits, and cattle. Host resistance has been shown to be a systemic immune response with humoral, cell mediated and complement dependent effector mechanisms. The resistance response as manifested by guinea pigs has many of the characteristics of a cutaneous basophil hypersensitivity response. Preliminary evidence shows that bovine responsiveness to tick infestation is the same type of reaction. Experiments to investigate resistance to louse infestation have centered on the louse-mouse association. Mouse resistance to lice is a response limited to the site of previous infestation and appears to possess some immune response components.

Over the past year, studies directed at characterizing the nature of mouse resistance to louse feeding have been carried out using several approaches. Immunosuppressants with known modes of action have been administered to the host at different times during the course of louse infestation. The effects on the louse burden and histology of the resistance response have been monitored. It has been found from these studies that alteration of the immunocompetence of the host delays and/or reduces the degree of resistance expressed and that the sequential appearance of cells associated with the host reaction were altered. Athymic mice have been used as recipients of skin grafts from sites on donor mice that have shown either resistance or susceptibility to louse feeding. These studies have shown that transfer and establishment of the graft on an animal devoid of a functional T-cell system does not alter the susceptibility of the transferred skin site to support a louse population. Investigations of the capability of a number of mouse strains to develop resistance to louse infestation has revealed a genetic basis for the response, with some strains developing a vigorous resistance while other strains display a constant high louse burden which represents the "carrier state." These differences in the ability to develop and express resistance are also evident in any population of randomly bred mice. Studies involving the mating of louse susceptible and resistant animals have been initiated in

an attempt to characterize the inheritance of these different resistance responses. Results of these experiments are just becoming available.

Research on the nature of mouse resistance to louse infestation will be reduced due to the forthcoming retirement of Dr. Bell; however, the above listed studies will be carried to completion. Adoptive transfer studies involving the passive transfer of viable lymph node cells and serum will be performed in an attempt to determine the role, if any, of these immune effector components in the resistance response. If resistance can be adoptively transferred, the particular components involved in the transfer of resistance will be characterized.

It has been determined that guinea pigs acquire resistance to larvae of D. andersoni after one infestation. Resistant animals allow significantly fewer larvae to engorge during a second infestation than during an initial exposure. Previous studies from this laboratory have revealed that the resistance response is composed of humoral, cell-mediated and complement dependent effector mechanisms. A fraction of salivary glands of adult, female D. andersoni has been shown to elicit antigen specific delayed skin reactivity in resistant guinea pigs and to induce an antigen specific in vitro proliferation of lymph node cells from resistant guinea pigs. Cattle made resistant to infestation with adult D. andersoni show similar skin reactivity and stimulation of their peripheral blood lymphocytes by this salivary gland antigen. This antigen, when combined with appropriate adjuvants, can induce a significant degree of tick resistance in guinea pigs not previously exposed to tick infestation.

Studies completed during the past year have demonstrated that the alternate pathway, rather than the classical pathway of complement activation is important in the expression of resistance. Experiments using various antihistamines with specificity against H_1 and H_2 histamine receptors have revealed that histamine is not apparently essential for the expression of guinea pig resistance to D. andersoni infestation. However, it appears that histamine might play a role in setting up an "itch-scratch" response at the site of tick attachment since the level of histamine, as measured by biochemical extraction, at the tick feeding site on resistant animals is five times that of normal tissue. It was hypothesized that the resistance response might play a role in the alteration of the vector capability of the blood feeding tick. It has now been established that the spread of highly virulent type A, Francisella tularensis can be blocked by host resistance to the feeding of infected ticks. This finding is of great practical importance. The role of the Langerhans cell in the resistance response has also been examined. This "reticuloepithelial" cell possesses many similarities with the macrophage and it has been shown that tick antigens were trapped by this cell in the epidermis. We have shown that cross-resistance to tick infestation does develop within the genus, but not across the genus barrier for ixodid ticks.

Experiments designed to further characterize the nature of tick resistance are in progress. The preliminary observations that ticks induce a degree of immunosuppression in the host during infestation is being examined by several approaches aimed at examining the humoral and cell-mediated immunocompetence of the tick-infested host. Studies are also designed to examine the generation of suppressor cells in the tick-exposed host. The role of tick resistance in the spread of pathogens from the vector to the host will be continued with the use of rickettsia-infected ticks in an attempt to determine if the transmission of Rocky Mountain spotted fever can be altered by the resistance response. The role of the Langerhans cell in tick resistance will be further examined by the use of ultrastructural immunocytochemical techniques to determine the fate of tick antigens during the course of an initial and second infestation. This study will be combined with a general ultrastructural characterization of the tick feeding lesions. Studies on cross-resistance will be continued utilizing the many genera and species of ixodid ticks in the RML colony.

Studies in conjuction with the U.S. Livestock Insects Laboratory of the U.S. Department of Agriculture will focus on the immune response characterization of cattle infested with the ixodid ticks D. andersoni and Boophilus microplus. These studies are designed to ultimately develop a means of immunomanipulating cattle to prevent tick infestation. Initial studies to characterize bovine immune responsiveness to ticks are in progress.

Publications:

Wikel, S. K., Graham, J. E., and Allen, J. R.: Acquired resistance to ticks. IV. Skin reactivity and in vitro lymphocyte responsiveness to salivary gland antigen. Immunology 34: 257-263, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00084-09 RML

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Vector-Pathogen Relationships

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. E. Yunker	Scientist Director	RML NIAID
OTHER:	J. Kurz	NIH Visiting Fellow	RML NIAID
	E. Ribi	Res. Chemist (Phys. Chem.)	RML NIAID

COOPERATING UNITS (if any) Dr. Kouka S.E. Abdel-Wahab, Al Azhar University, Cairo, Egypt; Dr. Mamdouh Y. Kamel, National Research Center, Cairo, Egypt; Dr. J. F. Anderson, Connecticut Agricultural Experiment Station, New Haven.

LAB/BRANCH
Rocky Mountain Laboratory, Hamilton, MT 59840

SECTION
Medical Zoology and Zoonotic Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 6/12	6/12	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project deals with the interactions of vector-borne microorganisms, arboviruses, rickettsiae and protozoans, and cells of haematophagus arthropods, mainly ticks. Model in vitro systems are developed and applied to studies of pathogen host-range, growth dynamics, persistence and interference. After prolonged growth in these systems, pathogens are examined for changes in invasiveness, virulence, or antigenic characteristics. By contract with collaborative laboratories overseas, we are studying critical factors influencing survival of ticks and of pathogens within ticks from a biochemical, histochemical, and physiological standpoint.

PHS-6040
(Rev. 10-76)

Project Description:

ALSA, an Alphavirus we found to be persistently infecting some strains of cultured mosquito cells was studied further. This virus, originally latent in these cells, can be expressed by stress or by subinoculation into uninfected cells. Infected cells significantly resist challenge of related viruses, Chikungunya (CHIK) and O'nyong-nyong, but not of other unrelated viruses. This year we found that mosquito cells that had recovered from ALSA virus infection were markedly resistant to challenge with CHIK virus, indicating that the former agent may have reverted to latency. This suggests the possibility that proviral DNA had become incorporated into the cellular genome. Alternatively, it is possible that ALSA exists as defective interfering particles.

A study of transovarial transmission of Flaviviruses by ticks was begun. The nature of viral nucleic acid during transovarial passage has been questioned by Soviet workers, who have identified DNA transcripts of arboviral RNA in the eggs of Hyalomma ticks infected with an unclassified virus, Bhanja. Our studies involve Dermacentor ticks and Langat virus as a model system. Parenterally inoculated female ticks were fed on rabbits. It was established by immunofluorescent (IF) test that hemocytes were heavily infected with virus, and that eggs also contained infectious virus. Tests of hatched F_1 larvae are in progress.

Attenuation of Colorado tick fever virus after prolonged arthropod cell passage is under study. Previously, we reported that prolonged growth in mosquito cells lessened virulence and increased the predominant plaque size of Florio strain CTF virus. It was suggested that selection of larger, less virulent plaque-variants might have been effected in the arthropod cells. This year, in another experiment employing cloned small-plaquing particles, we again demonstrated loss of virulence after prolonged growth in Aedes cells. However, plaque size was not altered. This suggests that a potential for attenuation is present in the virion regardless of plaque morphology and that some other mechanism, such as temperature sensitive mutation or variation, is operative.

Babesia microti, causal agent of human piroplasmosis in Massachusetts, was separated from host cell components in highly refined form by means of the Ribi-Sorvall cell fractionator. The material was used as an antigen in developing an indirect micro-IF test. This test is both reproducible and, unlike current IF tests for babesiosis, devoid of cross-reactivity with other piroplasms. (Tested were antisera from six species of Babesia, Anaplasma marginale and Plasmodium berghi.) A manuscript describing antigen production and testing is in preparation. The chemical composition of the B. microti antigen was investigated at various stages of extraction. Findings suggest that most of the compounds responsible for specific antibody production are carbohydrates and that species-specific differences exist among B. microti and other babesias. The IF test was

successfully employed in detecting babesial antibodies in rodent populations of Connecticut. Results are in manuscript.

Attempts were continued to repeat our recent singular success in establishing a serially propagating line of tick cells. Most promising to date are cultures prepared by inoculation of a tick-borne virus into primary cultures of Ornithodoros coriaceus cells. In contrast to uninoculated cells, which died shortly after explantation, virus-infected cells formed dense clumps surrounded by cellular outgrowths, while continuing to produce virus for over 2 months.

As in previous years, our specialized area of research has prompted requests from various federal, state, and university laboratories for arthropod cells and serological reagents, as well as for short-term training of personnel in arthropod tissue culture techniques.

We will continue to explore the nature of the pathogen-vector relationship at the cellular level. The success of a parasitic microorganism is often determined by its ability to exist in chronic or latent form. Arboviruses are most successful in cells of their invertebrate hosts, and it is this interface—not that of virus-vertebrate host—that insures their survival. Arboviruses in vector cells exhibit all known mechanisms that have been postulated to explain the development of persistent infections: defective interfering particles, temperature-sensitive mutation, and RNA → DNA transcription. Studies of these mechanisms may provide insight as to why vertebrate pathogens are able to replicate in vector cells with little or no cytopathology. An effective way to approach these studies would be from a standpoint of understanding viral RNA speciation and viral-specificed proteins produced in vector cells as a result of infection. We are not prepared to undertake such studies and recommend the addition to the project of a staff fellow with interests and training in molecular virology. We will continue to characterize patterns of viral and rickettsial growth in arthropod or other cell systems, with emphasis being placed on changes in virion properties (e.g., invasiveness, pathogenicity, interference). As one of the few infectious disease laboratories in the world with long experience in vector-cell culture, we will continue to explore the value of these systems for primary isolation, identification and characterization of pathogens, as well as for the production of antigenic material from these organisms.

Publications:

Bhat, U.K.M. and Yunker, C. E.: Establishment and characterization of a diploid cell line from the tick, Dermacentor parumapertus Neumann (Acarina: Ixodidae). J. Parasitol. 63: 1092-1098, 1977.

Publications: (cont'd)

In press:

Bhat, U.K.M. and Yunker, C. E.: Susceptibility of a tick cell line (Dermacentor parumapertus Neumann) to infection with arboviruses. Proc. 2nd International Symposium on Arctic Arboviruses, Mont Gabriel, Que., Canada, May 26-28, 1977.

Appendix 1. PL-480 Project 03-030-N. In vitro and in vivo Studies of Certain Tickborne Viruses in Vector and Host Cells, Al Azhar University, Cairo, Egypt.

Total Man Years: 16 3/12 Professional: 1 3/12 Other: 15

Annual Funding - $123,150.60

The first year of this renewed project, which was designed to study the comparative patterns of replication of typical tick-borne viruses pathogenic for man in vector and vertebrate-host tissues, was completed. Current research findings include identification of Quaranfil (QRF) virus antigens in host cell membranes, effect of ions on QRF virus yield, demonstration of temperature sensitive variants of QRF virus, and distribution of QRF antigens in tick tissues. Four papers are in press, one is in manuscript form, and two are in preparation.

Appendix 2. PL-480 Project 03-051-N. Biochemistry and Physiology of Tick Vectors of Disease.

Annual Funding - $218,858.00

This project is in its first year at the NRC. Its goal is to provide basic biochemical information necessary for: 1) the understanding of environmental factors to which pathogens are subjected when ingested by tick vectors, and 2) progress in development of tick tissue culture. Results are not yet available.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00085-01 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biology of Aleutian Disease Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. E. Bloom	Medical Officer (Res.)	RML NIAID
OTHER:	J. E. Coe	Medical Officer	RML NIAID
	R. E. Race	Veterinary Officer	RML NIAID
	W. J. Hadlow	Res. Veterinarian (Path.)	RML NIAID

COOPERATING UNITS (if any) None

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SECTION

Allergy and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 7/12	10/12	9/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this new project is to study Aleutian disease, a severe fatal immune complex disease of mink associated with a persistent viral infection. The project is a concerted effort and will focus techniques of immunology and virology on Aleutian disease. The current objectives are to characterize Aleutian disease virus (ADV) by standard virological methodology and to determine what role viral components have in the genesis of the immune complexes. Additional objectives are to study the immunoglobulin class-specific antibody response to ADV and to ascertain if severe immune complex disease correlates with a specific immunoglobulin class of antiviral antibodies.

PHS-6040
(Rev. 10-76)

Project Description:

Previous work at RML by this PI and others showed that the Utah I strain of Aleutian disease virus (ADV) could be successfully purified from in vivo grown materials by equilibrium ultracentrifugation in cesium chloride density gradients, if virus was first released from naturally occurring immune complexes. These studies suggested, on the basis of size, morphology, and density that ADV was probably a member of the parvovirus group, small icosahedral viruses that encapsidate single stranded DNA genomes.

Recently, Utah I ADV has been adapted to growth in cell cultures of Crandall feline kidney cells (CRFK) at 31.8°C and assayed, using immunofluorescence. We have successfully passaged this strain of ADV in CRFK at RML and are initiating a variety of studies.

Characterization of Utah I ADV. We have undertaken a detailed study to characterize ADV grown in CRFK cells by standard techniques and to compare in vivo and in vitro grown virus. Preliminary in vitro data have shown that ADV will replicate to high titer in vitro, is strongly cell associated, and displays one-hit kinetics of infectivity over a wide range of input multiplicity. Immediate plans are to define the infectious cycle, to purify virus, and to label the viral genome with appropriate nucleic acid precursors. These studies should allow definite assignment of ADV to the parvovirus family.

Production of anti-ADV antibodies in rabbits. In direct collaboration with Dr. John Coe, RML, we have prepared in rabbits an antiserum against in vivo grown, cesium chloride gradient purified ADV and are presently comparing antigenic specificities of this serum with sera of infected mink. The rabbit antiserum and infected mink sera form a single line of identity against ADV antigen in counter-immunoelectrophoresis, and the rabbit antiserum can successfully block direct viral immunofluorescence reactions in infected CRFK cells.

Future plans are to characterize viral polypeptides, to develop additional antisera against these proteins and to use these antisera to determine what viral components are present in both soluble and insoluble immune complexes.

Analysis of immunoglobulin (Ig) classes of anti-viral antibodies. An extensive study of anti-viral antibodies from infected mink has been precluded to date because of lack of a suitable assay system. With the capability of preparing replicate infected cell cultures, it will now be possible, using antisera to specific mink Ig classes, to analyze sera from infected animals and to observe if severe immune complex disease correlates with the presence or absence of a specific class of antiviral antibody. These studies will further allow comparison of class-specific antibody

response to ADV in persistently and nonpersistently infected mink. Such studies will utilize serological reagents and techniques developed by Dr. John Coe, RML.

Publications:

In press:

Bloom, M. E.: Slow virus diseases. In Feigin, R. and Cherry, J. (Eds.): Textbook of Pediatric Infectious Disease. Philadelphia, W. B. Saunders.

Bloom, M. E. and Rose, J. A.: Transcription of AAV RNA in KB cell nuclei. Virology

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00086-01 RML

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Dynamics of Tumorigenesis in Murine Leukemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Portis	Medical Officer (SURG)	RML NIAID
OTHER:	B. W. Chesebro	Medical Officer (SR SURG)	RML NIAID

COOPERATING UNITS (if any) None

LAB/BRANCH

Rocky Mountain Laboratory, Hamilton, MT 59840

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Allergy and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of this project is the study of mechanisms of host defense against virus-induced transplantable neoplasms. We are utilizing as a model the murine leukemia tumor FBL-3. This tumor, originally induced by Friend virus in a C57BL/6 mouse, has been found in vivo to undergo rapid and predictable changes in malignant potential. Ascites cells harvested at 7 days produce transient s.c. tumors that are rejected. Ascites tumor cells harvested late (14 days) produce progressive lethal tumors with widespread lymphogenous and visceral metastasis. Using these two cell populations and clones therefrom, we have compared the expression of membrane antigens, production of oncornavirus, and in vivo and in vitro immunogenicity. These studies are directed at gaining an understanding of the cellular mechanisms of altered behavior in vivo and defining the membrane structures required for successful immune recognition and elimination by the host. The primary immune response to the tumor cell inocula is currently being studied to define the early events in tumorigenesis that determine the ultimate behavior of the neoplasm.

Project Description:

1. Definition of the tumor system. We have been studying a murine leukemia tumor, FBL-3, which has the potential for rapid predictable change in its malignant potential during i.p. growth. This tumor was originally induced in a C57BL/6 mouse by Friend virus (FV) and expresses only the helper virus (LLV). The tumor cells express antigens recognized by anti-H-2^b and anti-FV sera. It has thus far not been classifed and lacks Ia8, Thy 1.2 antigens and membrane Ig. The tumor is generally maintained by ascites passage but has also been adapted to in vitro growth. We have observed that in vitro grown cells produced small tumors (<1.5 cm) when injected s.c. into C57BL/10 mice and the tumors are uniformly rejected by day 20 to 40. Passage of the in vitro cells i.p. for 7 days (early ascites) yields tumor cells with similar s.c. growth characteristics. However, passage of in vitro cells for 14 days (late ascites) results in tumor cells which, when injected s.c., produced progressive lethal tumors in 50 to 70% of recipients, accompanied by both lymphogenous and hematogenous metastases. The magnitude of this change in growth potential is illustrated by the finding that mice rejected up to 40 X 10^6 early ascites cells whereas only 500 late cells produced progressive tumors in 75% of recipients (a difference of $\geq 10^4$).

Initial studies sought to define the influence of non-neoplastic donor cells accompanying the tumor cells in the ascites population on the pattern of s.c. growth of this neoplasm. Dilution of non-neoplastic host cells by short term (1 week) passage of ascites cells in vitro had no effect on the behavior of either early (regressor) or late (progressor) ascites populations. Another technique to deplete donor cells was the i.p. passage of FBL-3 (H-$2^{b/b}$) in B10.A X A.BY (H-$2^{a/b}$) recipients. This allowed selective killing of host cells with anti-H-2^a plus complement. However, this too had no effect on the s.c. behavior of early and late tumor cells confirming the lack of participation of non-neoplastic donor cells in the change in malignant potential of FBL-3. The observed change thus appears to be a property of the tumor cells themselves.

In order to determine if the change in behavior of this tumor was the result of emergence of a variant or due to changes at the cellular level, we cloned the original in vitro grown tumor cells and both early and late ascites cells and tested the clones for s.c. growth potential. The majority of clones behaved like early cells (i.e., they were rejected). However, we now have one stable progressor which was derived from late ascites cells. Currently, we are testing clones of the parent population to determine if the variant detected in the late population pre-existed or arose de novo.

2. Properties of early and late tumor cells. The mechanisms responsible for the change in growth potential in vivo are currently under study. Initially, early and late ascites cells and clones therefrom

were compared for the membrane expression of antigens (H-2K^b and H-2D^b tumor specific) which may be required for immune recognition by the host. Using serologic techniques, no quantitative differences in the expression of these antigens have been noted. Recent reports suggest that the antigens responsible for tumor rejection in vivo may be different than those recognized by serology. We have recently developed a T lymphocyte proliferation assay which is being used to quantify antigen-specific recognition of tumor-associated antigens in vitro. No significant differences have been observed in the capacity of early and late ascites cells to stimulate blastogenesis of primed syngeneic splenic T lymphocytes.

A third parameter being studied is the production of oncornavirus by various regressor and progressor cell lines. Measurement of reverse transcriptase activity (related to total virus particles) and an infectious center assay (to measure infectious units) have been used to quantify virus production. All cell lines thus far tested produce moderate amounts of virus, ranging from 500 to 2000 pM/10^6 cells/hr incorporated ^{3}H-dTTP (reverse transcriptase assay), and from 7 to 10 X 10^2 PFU/10^6 cells (infectious center assay). Although no significant quantitative differences in virus production have been observed between regressor and progressor FBL-3 populations, qualitative differences, such as immunosuppressive capacity, are currently being examined. Recent evidence by other workers strongly suggests that both the entire FV complex and the helper virus have immunosuppressive properties in vivo and in vitro. However, there is extensive heterogeneity between virus clones with respect to immunosuppressive potential. We are presently quantifying the in vitro immunosuppressive properties of FBL-3 virus from early and late ascites and from cloned cell lines.

3. Host response to early and late ascites tumor cells. It is clear that the host's immune response to the tumor is important in determining the growth characteristic of early and late ascites cells. Sublethal irradiation (450r) of recipients of early tumor cells results in the production of progressive rapidly lethal tumors. This indicates that there is no intrinsic limitation on growth of early cells in the subcutis. In addition, we have shown that animals that have previously rejected an inoculum of early ascites cells are then capable of rejecting late cells given either s.c. and i.p. This confirms data from the serologic and T-cell recognition studies, indicating that late ascites cells express the antigens required for tumor rejection.

The s.c. growth curves for early and late tumor cells show that both populations grow at comparable rates for 10 to 14 days, whereupon early cells are rejected and late cells produce progressive tumors. Thus, the first 10 days of tumor growth appear to be critical in determining the ultimate fate of the tumor. Studies are in progress to assess the

kinetics of the primary immune response (as measured by cell mediated cytotoxicity) to early and late tumor cells. Preliminary data suggest a measurable delay in the generation of cytotoxic lymphocytes in the lymph nodes draining the tumor bed and in the spleens of animals inoculated with late ascites cells s.c.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00087-01 RML

PERIOD COVERED: October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunological Activity of Eukaryotic and Prokaryotic Cellular Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Cantrell	Senior Staff Fellow	RML NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	RML NIAID
	C. A. McLaughlin	Senior Staff Fellow	RML NIAID
	K. B. Von Eschen	Staff Fellow	RML NIAID

COOPERATING UNITS (if any) Dr. G. F. Springer, Evanston Hospital, Evanston, IL; Dr. R. Wheat, Duke University Medical Center, Durham, NC; Dr. J. J. Killion, Oral Roberts University, Tulsa, OK; Dr. R. Tuttle, Burroughs-Wellcome, Research Triangle Park, NC.

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of this project is to evaluate the immunological activity of defined microbial components and isolated tumor-associated antigens that are efficacious antitumor agents. In the past year, attempts were made to 1) evaluate cell-mediated immunity in animals cured of neoplastic disease with microbial components, 2) partially purify tumor-associated antigens from tumor cells and ascites fluid that have antitumor activity, and 3) isolate the active component(s) from Corynebacterium parvum responsible for tumor regression.

PHS-6040
(Rev. 10-76)

Project Description:

Previous studies have shown that nonspecific immunostimulants were effective in treating line-10 tumors transplanted into the skin of syngeneic guinea pigs. Examples of such stimulants are aqueous soluble extracts isolated from delipidated whole cells of Bacillus Calmette-Guérin (BCG) or from whole cells of Re mutants of Salmonella typhimurium. Successful therapy was possible only upon proper combination of these nonspecific immunostimulants with trehalose dimycolate (P3) in an oil-in-water emulsion and administration by the intratumor route. Animals cured of tumors developed systemic tumor immunity as measured by their ability to reject a challenge of line-10 tumor cells. Additional studies were made to evaluate cell-mediated tumor immunity in guinea pigs following intratumor injections of these emulsions. Spleen cells from treated animals were tested for their influence on tumor growth by means of an in vivo adoptive neutralization test (Winn test). The results showed that the passive transfer of spleen cells from cured animals inhibited tumor growth in normal guinea pigs if the immune spleen cell to tumor cell ratio was 200:1 or greater. This transfer also resulted in the development of tumor immunity in that recipients of the cell transfer were able to reject a rechallenge of a lethal dose of tumor cells. No inhibition of tumor growth was observed in animals given spleen cells from nonimmune or tumor-bearing control animals. In addition, the antitumor activity of spleen cells from immune donors was abolished following pretreatment with T-cell specific antisera plus complement, whereas no significant reduction in antitumor activity was seen with immune spleen cells treated with B-cell specific antisera. These results indicate that cell-mediated tumor immunity is elicited in guinea pigs cured with emulsions containing P3 combined with aqueous-soluble extracts from BCG or Re mutant Salmonella species.

Recent studies in this laboratory have been devoted to characterizing the in vivo biological and antitumor activities of toxic and nontoxic components isolated from the Re mutants of S. typhimurium in mice models. This will allow us to more rationally predict the immunopotentiating activity of these microbial agents. A positive correlation was found between the ability of endotoxic extracts to bring about regression of line-10 tumors in guinea pigs and their ability to induce spleen enlargement and an increase in mitogenic activity in endotoxin responder mice. Conversely, nontoxic extracts had no antitumor activity in guinea pigs which correlated with normal spleen indices and mitogenic activity in mice. Of interest was the finding that C57BL/10 mice, syngeneic with C57BL/6 mice at the major histocompatibility complex, were completely nonresponsive to endotoxin extracts.

The antitumor activity of extracts from Re mutants of S. typhimurium are being evaluated in endotoxin responder and nonresponder mice with established EL-4 lymphomas. Results of these studies showed a marked increase in the incidence of tumor regression in responder mice treated

with chemotherapy combined with endotoxin immunotherapy, whereas no tumor regression was seen in responder animals given immunotherapy only. In contrast, no tumor regression was observed in nonresponder mice given combination immunotherapy and chemotherapy. These results suggest that the antitumor activity of endotoxin in murine tumor models is dependent, in part, on the host's ability to respond to the immunostimulant and that this response is genetically controlled.

Nonviable whole cells of Corynebacterium parvum are effective in treating a carcinogen-induced leukemia (L-1210) in syngeneic mice. In collaboration with Dr. Wheat, Dr. Killion, and Dr. Tuttle, studies have been devoted to isolating and characterizing the active component of phenol-water extracts of C. parvum. Tumor regressive activity was detected in the phenol insoluble residue fraction in mice given combination chemotherapy and immunotherapy. Tumor regression was dependent on pretreating the animal with chemotherapy prior to immunotherapy. In addition, a positive correlation was made between the fraction having antitumor activity and its ability to induce splenomegaly and to induce mitogenic activity. When this fraction was treated with periodate, the biological and antitumor activities were suppressed. These results suggest that the probable active component in this fraction is carbohydrate in nature, but periodate sensitive peptides also have to be considered. Recently, two discrete and easily separable fractions (light and heavy) have been isolated from this residue and studies are in progress to determine their antitumor activity.

Another area of interest to us is the use of specific immunostimulants in the regression of established tumors. Since most tumors, regardless of etiology, have tumor-associated antigens on their cell membrane, a series of experiments were designed to determine the value of solubilized tumor antigens (KCl extracts of tumor cells) in causing the complete regression of EL-4 lymphoma in C57BL mice. High cure rates (80%), which were significantly higher than those of chemotherapy-only control groups, were observed in animals given combination chemotherapy and immunotherapy. Successful therapy was dependent on emulsifying the soluble tumor antigen in minute oil droplets. No tumor regression was seen in animals given chemotherapy followed by an injection of tumor antigens solubilized in saline. Emulsions containing tumor cell extracts, when tested without chemotherapy, had no therapeutic value. Animals cured by combination chemoimmunotherapy had detectable tumor-specific immunity. Moreover, the cytotoxic activity of sera from cured mice was tumor specific. These results demonstrate that it is possible to isolate tumor-associated antigens which are immunogenic and which possess high tumor regressive potency when combined with intratumor chemotherapy. These findings were reported at the Annual Meeting of the American Association for Cancer Research, April 5-8, 1978, in Washington, D.C.

Inquiries were also made this year into the possible isolation of tumor-associated antigens from the ascites fluid of strain 2 guinea pigs with progressively growing line-10 ascitic tumors. Butanol-water extracts of ascites fluid yielded multiple fractions, and tumor-associated antigens were found in the butanol phase. To test the antitumor activity of these extracts, we injected oil-in-water emulsions containing P3 combined with the ascites extracts directly into the dermal tumors. Antitumor activity was found only in the antigen-containing butanol fraction. In animals pretreated with intratumor chemotherapy, followed by intratumor injection of tumor antigen, a higher incidence of tumor regression was observed. Animals cured with the butanol phase of ascites fluid had systemic tumor immunity as measured by delayed type hypersensitivity to tumor cells and isolated tumor antigens, and their ability to reject a rechallenge of line-10 tumor cells.

Experiments are in progress, in collaboration with Dr. Springer, to determine the cross-reactivity between line-10 tumor cells and microbial extracts with human blood group antigens. Preliminary results indicate that line-10 cells have a substance that cross-reacts with human MN antigens in addition to a substance that cross-reacted with human B antigen. Strain 2 guinea pig cells only cross-react with human B antigen. Similar findings were observed with the isolated tumor antigens from the ascites fluid. In addition, this same cross-reactive antigen was detected in the endotoxin extracts isolated from Re mutants of S. typhimurium. These experiments are part of a long-range plan to study the cross-reactivity between microbial components having antitumor activity and neoplastic cells. Such an approach would be supplemented by treatment with combinations of tumor-associated antigens and immunopotentiating microbial components in an attempt to design the most efficacious tumor therapy.

The future course of this project will include studies designed to evaluate cellular and humoral immunity in animals cured of neoplastic disease with soluble tumor-associated antigens. Immunoprophylactic studies are now in progress, using isolated tumor antigens alone or in combination with tumor cells treated in vitro with mitomycin C. Both humoral and cellular immunity will be evaluated in these animals to determine the immunological profile that correlates with successful therapy. In addition, attempts will be made to further purify and characterize tumor-associated antigens isolated from guinea pig and murine tumors. These refined fractions will be tested for their antitumor activity alone or in combination with microbial components that bring about tumor regression. Hopefully, we will better understand the mechanism(s) of tumor regression and we will be able to design immunotherapy protocols which are the most potent and least harmful. Collaboration with Dr. Wheat, Dr. Killion, and Dr. Tuttle will be continued in attempts to isolate the antitumor component from C. parvum. Animals treated with this antitumor agent will be evaluated for tumor specific immunity. In addition, collaboration with Dr. Springer will be continued in attempts to isolate

the antigen(s) found in microbial and tumor extracts that cross-react with human blood group antigens. Only if this is accomplished will we be able to determine the efficacy of these antigens in tumor regression.

Publications:

Cantrell, J. L., Ribi, E., and McLaughlin, C. A.: Passive transfer of tumor immunity with spleen cells from guinea pigs cured of hepatocarcinoma by nonspecific immunotherapy. Cancer Immunol. Immunother. 4: 21-28, 1978.

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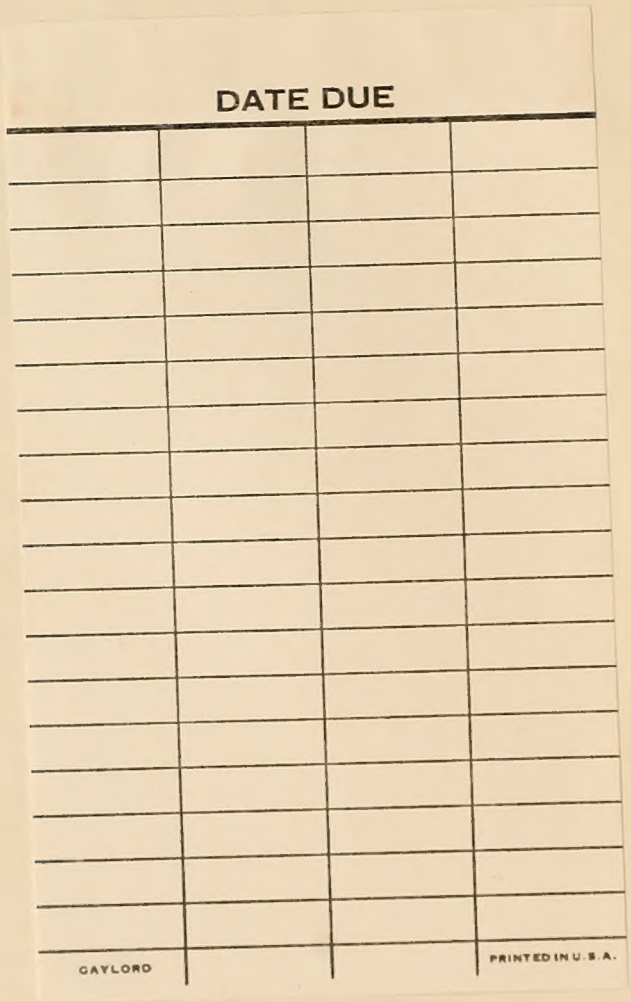

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